Effect of VEGF on the Revascularization of Severed Human Dental Pulps

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ABBREVIATIONS

Commonly Used Abbreviations

VEGF	Vascular Endothelial Growth Factor, $VEGF_{165}$,VEGF-A
bFGF	Basic Fibroblast Growth Factor, FGF-2, Fibroblast growth factor-2
mins	Minutes
EC	Endodothelial Cell
ETOH	Ethanol
DDW	Distilled Water
ref.	Reference No.
#	Number
RT	Room Temperature
Ab	Antibody
CEJ	Cemento-enamel junction
SCID	Severe Combined Immunodeficient
EPCs	Endothelial Progenitor Cells
RGB	Red Green Blue
TIFF	Tagged Image File Format
PDGF	Platelet Derived Growth Factor
PlGF	Placental Growth Factor
EGF	Epidermal Growth Factor
LPS	Lipopolysaccarhide
LTA	Lipoteichoic acid

IL	Interleukin
TNF	Tumor Necrosis factor
IFN	Interferon
MMP	Matrix Metalloproteinase
PDL	Periodontal ligament
DMEM	Dulbecco's Modified Eagle Medium
IHC	Immunohistochemical
UCUCA	University Committee on Use and Care of Animals
IRB	Institutional Review Board

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SECTION I

1. BACKGROUND AND SIGNIFICANCE

The most common injuries to the permanent dentition are due to falls, traffic accidents, acts of violence and sports (Andreasen *et al*., 2000). Avulsion frequently occurs with injuries to the oral facial region and is the most serious of all dental injuries (Flores *et al*., 2007b). Treatment of these injuries is of the utmost importance and guidelines for the management of traumatic dental injuries have recently been published (Flores *et al*., 2007a, 2007b). Even with these guidelines and appropriate treatment of avulsed permanent teeth, the outcomes of replantation are not predictable (Strobl *et al*., 2003). It is often only considered a temporary solution (Ebeleseder *et al*., 1998). We have known for over 40 years that the avulsed immature tooth has potential to revascularize (Ohman, 1965). A treatment that encourages revascularization would be beneficial because in an immature tooth root development and reinforcement of root dentin walls by deposition of hard tissue will continue (Andreasen *et al*., 1995b). This decreases the chance of tooth fracture and avoids endodontic treatment which will ultimately weaken the tooth.

Pulpal revascularization of the replanted tooth involves anastomosis of severed pulpal and boney socket blood vessels. Angiogenesis, the process of formation of new blood vessels from pre-existing capillaries governs the revascularization of these teeth. It has been known for more than a century that tumor growth is accompanied by increased vascularity (Ferrara, 2004). Over sixty years ago it was proposed that a diffusible angiogenic growth "factor X" was responsible for neovascularization in the retina that occurs in diabetic retinopathy (Michaelson, 1948). Thirty years ago Folkman first proposed that angiogenesis was important for tumor growth and development (Folkman, 1971; Folkman and Shing, 1992;

Sivakumar *et al*., 2004). They discovered that the “switch” from avascular to the vascular phenotype was the key event in the progression of metastatic tumors (Hanahan and Folkman, 1996).

It is now known that VEGF is a key player in angiogenesis and approximately three years ago, clinical trials were conducted which involved direct intramyocardial plasmid $VEGF_{165}$ gene therapy in patients with stable severe angina pectoris (Kastrup *et al*., 2005). $VEGF_{165}$ gene transfer did not significantly improve myocardial perfusion but it improved regional ventricular wall motion (Kastrup *et al*., 2005). The results were promising and $VEGF_{165}$ gene therapy is thought to have an important future role to play in the treatment of myocardial disease.

Recently a tooth-slice based *in vivo* model system for the study of revascularization of severed human dental pulp was developed. It was shown that $rhVEGF_{165}$ induced angiogenesis in dental pulps of human tooth slices *in vitro* (Gonçalves *et al*., 2007). With this model it is now possible to study revascularization events in severed human dental pulp. We also have the opportunity to study the effects of $rhVEGF_{165}$ on severed human dental pulps *in vivo*. Our present study may have implications on the treatment of avulsed immature teeth prior to replantation.

2. PURPOSE AND HYPOTHESIS

Purpose

To evaluate the effect of $rhVEGF_{165}$ and rhFGF-2 on the microvessel density of severed human dental pulps *in vitro* and to evaluate the angiogenic effect of the application of $rhVEGF_{165}$ on severed human dental pulps *in vivo*.

Hypothesis

A pro-angiogenic factor ($rhVEGF_{165}$) induces revascularization and angiogenesis of severed human dental pulps implanted in the subcutaneous tissue of immunodeficient mice.

Specific aims

Specific Aim 1: To assess the effect of the pro-angiogenic factors $rhVEGF_{165}$ and rhFGF-2 on the microvessel density of severed human dental pulp tissues *in vitro*.

Specific Aim 2: To evaluate the effect of the protocol that provided the most potent angiogenic response (from Specific Aim 1) on severed human dental pulp tissue *in vivo*.

Null Hypothesis

A pro-angiogenic factor ($rhVEGF_{165}$) does not induce revascularization and angiogenesis of severed human dental pulps implanted in the subcutaneous tissue of immunodeficient mice.

3. LITERATURE REVIEW

A. Replantation and Revascularization of Avulsed Immature Permanent Teeth

Dental injuries to permanent maxillary incisors are frequently found after orofacial trauma and treatment outcome is determined by the severity of damage to the periodontal ligament and neurovascular supply (Andreasen *et al*., 1995a, 1995b, 1995c, 1995d). Tooth avulsion is a complex type of trauma and involves damage to several vital structures (Andreasen *et al*., 2006). Replantation is the treatment of choice, but complications occur; ankylosis, pulpal necrosis, pulpal obliteration, external root resorption and loss of boney support (Andreasen *et al*., 1995a, 1995b). Outcomes of replantation may vary and may not be predictable from the appearance or extent of injury sustained clinically (Strobl *et al*., 2003). Due to unpredictable treatment outcomes, replantation is often considered a temporary solution (Ebeleseder *et al*., 1998). For the clinician it is difficult to determine whether replantation will have a satisfactory long-term result, because many factors remain unknown at the time of replantation. Therefore replantation is recommended in nearly all cases.

Factors that determine pulpal healing of avulsed teeth are the stage of root development at the time of injury and the time period between trauma and replantation (Andreasen *et al*., 1995b). The most successful outcome of replantation is pulpal healing, but if this is to occur, revascularization of the severed pulpal vasculature must occur (Strobl *et al*., 2003). This is difficult especially in cases of delayed replantation (Ebeleseder *et al*., 1998).

In 1966, Andreasen showed that of 13 teeth, seven showed pulpal revascularization when their extra oral period was less than two hours. Revascularization was also observed in 13 of 72 replanted teeth with incomplete root formation (Kling *et al*., 1986). There was also a 30-

40% chance of revascularization seen in replanted immature teeth (Ebeleseder *et al*., 1998). If all teeth were replanted immediately, a periodontal ligament healing rate of 85-97% (according to root development) could be expected. In cases of incomplete root formation, pulpal revascularization could be expected in 41-93% (Cvek *et al*., 1990) of cases and is rare in mature replanted teeth of children and adolescents with closed apices. More frequent pulpal healing was seen with increased apical diameter which is associated with the importance of a large interface between the pulp and periodontium in order to facilitate the revascularization process. This, in conjunction with immediate transplantation, showed significantly more frequent pulpal healing than delayed replantation cases (Andreasen *et al*., 1995b). Pulpal revascularization was more frequent in teeth with shorter distances from apical foramen to the pulp horns. The relationship between pulp survival and pulp length was found to be highly significant. Thus all teeth with shorter pulp (less than 17mm) showed more frequent pulpal revascularization (Andreasen *et al*., 1995b). Also wet storage media (saliva or saline) for more than 5 minutes decreased the chance of revascularization, but dry extra-alveolar storage decreased the chance of revascularization with increasing length of extra-alveolar dry storage. Dehydration of pulpal cells could be expected to occur to a more limited extent due to protection against drying by the root canal walls except at the apical foramen. The damaging effect of dry storage upon healing found by Andreasen might therefore be limited to the most peripheral part of the pulp in the apical foramen. This effect in theory could be related to problems in the initial phases of the revascularization process.

Attempts have been made to increase the chances of revascularization in order to gain more favorable treatment outcomes. Soaking avulsed dog teeth extra orally for 5 minutes in 0.05 mg/ml of doxycycline before replantation increased the frequency of revascularization from

33.4% to 60%. (Yanpiset and Trope, 2000). Topical treatment of avulsed immature dog teeth (extracted and replanted within 10 minutes) with minocycline microspheres (Arestin^{TM}, OraPharma Inc.) for 5 minutes was seen to improve the chances of revascularization after replantation when compared to doxycycline and saline. 91% of the teeth treated with minocycline showed vital tissue in the canal space. Normal pulp tissue with normal odontoblast layers were noted post histological sectioning. Internal reactive dentin with connective tissue, osteoid with loose connective tissue was also observed (Ritter *et al*., 2004). It appears from these studies that tetracycline activity had a positive effect on revascularization. Tetracycline's activity is related to its broad spectrum of activity against gram positive and gram negative bacteria. They also affect MMPs, which are a family of collagenases. The activity of MMPs appears crucial in the destruction of the major structural tissue protein, collagen. Tetracyclines inhibit collagenase activity and osteoclast function (Golub *et al*., 1991). They bind to tooth tissue and can be slowly released in active forms (Baker *et al*., 1983). Tetracyclines also promote the binding of fibroblasts to connective tissue thus enhancing PDL regeneration. But perhaps a more plausible explanation for tetracyclines effects on avulsed dog teeth is that, the necrotic pulp stayed free of bacteria and it could thus act as a scaffold for the tissue revascularization of the pulp space (Ritter *et al*., 2005). These studies confirmed the clinical benefit of doxycycline and minocycline to enhance the revascularization of replanted teeth, but one has to remember that these studies were not conducted on human teeth.

Prior to tetracyclines being used to treat avulsed teeth, attempts were made to increase the surface area of pulp tissue exposed to the bone-PDL interface. It was thought that this may increase the chances of revascularization. Apicoectomies were performed prior to

replantation. Teeth were extracted and replanted within 10 minutes and 2 mm of the apices of monkey teeth were resected for the purpose of examining pulpal vascularization (Andreasen *et al*., 1985). These teeth were examined histologically eight weeks after replantation and analysis showed that there was less vital pulp tissue found in teeth with immature root formation. Apical resection resulted in infection of pulp tissue, the apical tissues were damaged to such an extent that revascularization was slowed or made impossible and also resulted in a gap between the bone blood supply and the pulp. Similar results were seen for mature teeth. It was concluded that resection of the apices prior to replantation should be contraindicated (Andreasen *et al*., 1985). Unpredictable results were also observed using human teeth (Bolton, 1974; Janson *et al*., 1978).

Similar studies were conducted using dog teeth. Pulpal revascularization occurred in eight of nine apicoectomized teeth (extracted and replanted within 10 minutes) during a four month observation period (Skoglund, 1981a). Revascularization did not occur in the teeth which were replanted and autotransplanted with intact roots or mature roots. The revascularization capacity of the pulp in transplanted and autotransplanted teeth could be increased if an apicoectomy is carried out at the time of replantation (Skoglund, 1981a). Histological assessment was carried out on these eight revascularized teeth. It was observed that the pulp of mature non apicoectomized replanted teeth had necrosed. In the mature teeth that were apicoectomized the pulp had first necrosed and then underwent repair via cells from the apical bed. This resulted in the formation of connective tissue (scaring) in the pulp space and was later replaced by bone or cementum like hard tissue. This tissue would make endodontic treatment difficult at a later date. Survival of the original pulp tissue was not achieved (Skoglund, 1981b).

Further attempts to increase the chances of revascularization were made by adding nutritional canals to resected root tips of mature dog teeth. It was hoped that these canals, three in total with a diameter of 1 mm, would aid in revascularization. The preparation of these canals did not lead to more extensive revascularization of the pulp. Some of the vessels that were growing in from the apical area anastomized to vessels growing in from the nutritional canals, but no advantage was gained from the preparation of the canals (Skoglund, 1983).

The results from studies involving dog teeth must be interpreted with caution. Dogs have a peculiar root anatomy, with an apical delta which differs from humans which have an apical constriction. Thus resecting the apex removes the delta and makes a more favourable environment for pulpal healing (Andreasen *et al*., 1985).

Revascularization of avulsed immature teeth is possible, but it only occurs in a small percentage of cases. The achievement of revascularization is beneficial as the extent of root development is related to pulpal revascularization (Andreasen *et al*., 1995c) and neurovascular survival. The necrotic pulp is avoided, root development is continued resulting in increased tooth strength against root fracture due to continued odontogenesis (Banchs and Trope, 2004). An infection free pulp-space is maintained, inflammatory resorption is avoided (Strobl *et al*., 2003) and the expense and danger of a procedural iatrogenic error associated with endodontic treatment is circumvented (Mesaros and Trope, 1997).

B. Pro-angiogenic Factors

Angiogenesis is the process that results in the formation of new blood vessels that deliver oxygen and nutrients to cells and tissue (Sivakumar *et al*., 2004). Blood vessels may originate from two processes: vasculogenesis and angiogenesis. In vasculogenesis, endothelial cell differentiation occurs from mesodermal precursor cells (angioblasts), whereas, in angiogenesis, new vessels are formed from pre-existing ECs. Angiogenesis is an important part of many natural processes, embryonic development, ovulation wound repair and pathological processes, arthritis, diabetic retinopathy, and tumors growth. In many diseases, the body looses control of angiogenesis, resulting in an excessive blood vessel development, as observed in cancer (Folkman and Shing, 1992). Vasculogenesis occurs during embryonic development and leads to the formation of the primary vascular plexus. This is further developed, forming a more complex system and ramifies into larger and smaller vessels. New capillary vessels are then formed through angiogenesis by the division of the original vessels (Auerbach and Auerbach, 2001).

Angiogenesis is the result of a net balance between the activities of positive and negative regulators, survival and death signals (Nör and Polverini, 1999); proangiogenic and antiangiogenic factors. It is also a complex process and is controlled by a variety of growth factors, cytokines, proteases and protease inhibitors released from activated monocytes as well as ECs, smooth muscle cells, and platelets. It involves EC migration and proliferation, ECM breakdown, which is needed to provide space for enlarging vessels, pericytes, chemotaxis of macrophages, smooth muscle cell proliferation, development of new vascular structures and new extra cellular matrix deposition (Folkman, 1995).

Angiogenesis occurs when there is an increase in tissue size or an increased need for oxygen in hypoxia (Sivakumar *et al*., 2004). Apoptosis or programmed cell death is crucial to the angiogenic process. It is now established that key regulators of angiogenesis function, at least in part, by modulating the survival of ECs during the process of vessel repair and angiogenesis. Apoptosis is a mechanism that regulates the reshaping of tissues and organs (Fig. 1). It insures the survival of cells with the best adaptation to the environment, and eliminates infected, damaged cells that may have the potential to become neoplastic and thus when newly formed vessels are no longer needed they undergo regression. Targeted apoptosis of ECs seems to have an important physiological role allowing for the communication between the newly formed vessels and their parent capillaries (Nör and Polverini, 1999) (Fig. 1).

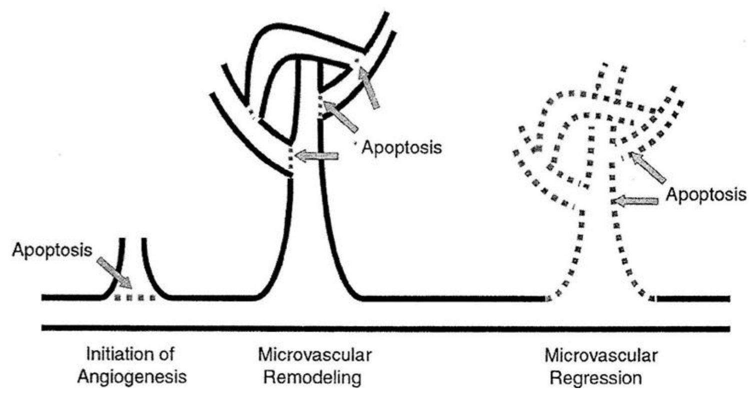

Figure 1. Apoptosis and Angiogenesis. (Nör and Polverini, 1999).

Growth Factors

Growth factors are peptide molecules that transmit signals between cells functioning as stimulators and or inhibitors of growth as well as modulators of cell differentiation. They play a central role in controlling cell behavior and activity. They show a degree of specificity in terms of the cells they act upon, but some growth factors exert their effects on a number of cell types (Smith, 2003).

Growth factors may act in endocrine, autocrine, paracrine modes and they act through their interaction with specific receptors on the cell surface (Smith, 2003). Binding to these receptors leads to a chain of intracellular signals, the result of which is transduction of the signal to the cell nucleus. It is through their effects on gene expression in the cell nucleus, mediated by transcription and other factors, that the growth factors influence cell behavior and activity. This transcriptional control of gene expression can have far-reaching effects both in terms of intra- and extra-cellular events and so growth factors can regulate genes controlling cell proliferation, cell differentiation, or the secretory products of the cell (Smith, 2003).

There are vast numbers of growth factors and the following are pro-angiogenic factors. The VEGF family includes VEGF-A (commonly called VEGF), VEGF–B, VEGF–C, VEGF–D, VEGF–E and PDGF (platelet derived growth factor). Other growth factors are EGF (epidermal growth factor), Angiopoietin-1 and Angiopoietin-2 (Ferrara, 2004), Hepatocyte Growth Factor, TGF (Transforming growth factor α and β) (Abo-Auda and Benza, 2003) (Table 1), FGF-2 and IL-8; these do not belong to the VEGF family.

Table 1. Superfamilies and families of commonly recognized growth factors

Superfamily	Family	Abbreviated name
Transforming Growth factor β	Transforming Growth factor β	TGF-β
	Bone Morphogenic Proteins	BMP
	Inhibins	Inhibin/Activin
Platelet-derived growth factor	Platelet-derived growth factors	PDGF
	Vascular endothelial growth factors	VEGF
	Connective Tissue Growth factors	CTGF
Epidermal growth factor	Epidermal growth factors	EGF
	Transforming growth factor α	TGF-α
Other large peptide growth factor families	Fibroblast growth factors	FGF
	Insulin-like growth factors	IGF
	Nerve growth factor	NGF
	Tumor necrosis factors*	TNF

*TNF-α and TNF-β are usually classified as pro-inflammatory cytokines, but sometimes considered within growth factor Classifications.

(Adapted from Smith, 2003)

Table 2. Specificity of the VEGF Receptors to the Ligand and the biological effects.

VEGF Family Members	Receptor	Function
VEGF-A	VEGFR-1, VEGFR-2	Angiogenesis, vascular maintenance
VEGF-B	VEGFR-1	Not established
VEGF-C	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-D	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-E	VEGFR-2	Angiogenesis
PlGF	VEGFR-1	Angiogenesis and inflammation

(Adapted from Grando-Mattuella *et al.*, 2007b)

Vascular Endothelial Growth Factor

VEGF, a 45-kd heparin-binding glycoprotein, induces the differentiation, proliferation, and migration of endothelial cells, as well as vascular permeability and cell survival. It is considered essential for the differentiation of the vascular system (Ferrara, 1996).

VEGF-A and VEGF-B are primarily related to angiogenesis (Table 2). VEGF is transcribed from a single gene locus by alternate splicing and it is made of polypeptide subunits containing 121, 145, 165, 189 and 206 amino acids. $VEGF_{165}$ is the most common isoform (Tischer *et al*., 1991). VEGF expression is mediated by hypoxia and VEGF driven angiogenesis is a central response to low oxygen tension and it involves transcription factors HIF-1 and HIF- 2 (hypoxia induced proteins).

VEGF receptors are primarily expressed on the vascular endothelial cell surface (Fig. 2). They include the tyrosine-kinase (RTK) type and are present in three forms: the fms-like tyrosine-kinase–1 (Flt-1) or VEGFR-1; the fetal liver kinase–1 (Flk-1), kinase domain region (KDR) or VEGFR-2; and the fms-like tyrosine-kinase– 4 (Flt-4) or VEGFR-3 (Pimenta *et al*., 2003). VEGFR-1 is found on smooth muscle cells (Ishida *et al*., 2001), Monocytes (Barleon *et al*., 1996), stem cells (Hattori *et al*., 2002) and bone marrow derived blood cells (Lyden *et al*., 2001). VEGFR-2 is found on EPCs. There is strong evidence that the three receptors for VEGF possess different signal transduction properties and mediate different functions because of their distinct affinity to VEGF (Keyt *et al*., 1996). Neuropilin is a type 1 membrane receptor, acts as a coreceptor that binds both $VEGF\text{-}A_{165}$ and $VEGF\text{-}B_{167}$. Neuropilin 1 may also potentiate the effects of VEGF-B in cells where VEGFR-1 is expressed (Makinen *et al*., 1999).

VEGF is a survival factor for endothelial cells and prevents apoptosis (Alon *et al*., 1995) by inducing antiapoptotic proteins Bcl-2 (Gerber *et al*., 1998; Nör and Polverini, 1999), by activation of the PI3k/Alt pathway. This mediates a potent pro-survival effect on ECs (Gerber *et al*., 1998). VEGF is required for maintaining ECs survival and to sustain tumor angiogenesis. When a positive survival signal is eliminated, ECs become responsive to inhibitors of angiogenesis leading to EC apoptosis and tumor regression. The events that govern the survival and death of ECs influence the stability and duration of an angiogenic response (Nör and Polverini, 1999).

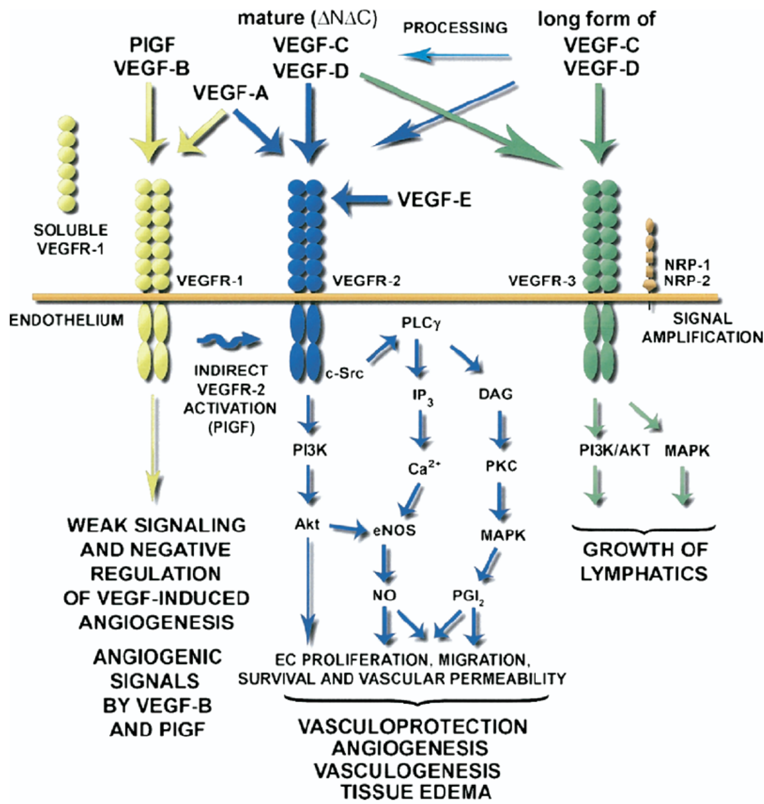

Figure 2. Binding of the VEGF Family Members to three High-Affinity Receptors on Endothelium and Downstream Signaling Cascades.

(Yla-Herttuala *et al.*, 2007).

Asahara *et al*. (1999) suggested that adult bone marrow is a reservoir of tissue specific stem and progenitor cells which contain EPCs that may be mobilized into the bloodstream and take part in neoangiogenesis. Depending on different diseases or events, molecules such as VEGF and PlGF have important roles in mobilization and differentiation of EPCs at neovascularization sites. The exact mechanisms by which EPCs are recruited remain unknown.

VEGF has the ability to promote growth of ECs derived from veins, arteries and lymphatics. VEGF also acts to increase vascular permeability and other hemodynamic effects. VEGF induces EC fenestration in vascular beds and induces vasodilation in a dose dependent fashion (Ferrara, 2004). VEGF released by monocytes and ECs act on the capillaries and in doing so VEGFR-1 and VEGFR-2 are upregulated on these cells. Their activation results in the generation of proteases such as plasmin and collagenase, which then dissolve the basement membrane (Dvorak *et al*., 1995). There is also upregulation of integrins at the tips of sprouting capillaries which enhances endothelial cell migration and receptor activation. This results in EC migration via VEGFR-1 (Dvorak *et al*., 1995) and EC proliferation via VEGFR-2 signaling (Plouet *et al*., 1997).

VEGF has a role in pathological conditions. VEGFR-1 is expressed by cancer cells which include glioma, leukemia, prostate, pancreatic and breast cancer cells. VEGFR-2 is primarily expressed on ECs. The inhibitory effects of anti-VEGF receptor blocking agents are used to inhibit angiogenesis, but this research is at an early stage and more clinical research is needed to ascertain the potential for these agents. Chemotactic signals from tumor cells recruit stromal cells and other angiogenic factors. Willet *et al* 2004 showed that VEGF

blockade using anti-VEGF monoclonal antibody bevacizumab (Genentech) decreases tumor perfusion, vascular volume, microvascular density, interstitial fluid volume, and the number of viable circulating ECs and EPCs in colorectal cancer patients. Bevacizumab (Avastin) was FDA approved in 2004 and it is now used to treat several cancer types including metastatic colorectal cancer and lung cancer.

Clinical trials have been conducted with direct percutaneous intramyocardial plasmid phVEGF-A_{165} injection in patients with severe coronary artery disease (Kastrup *et al*., 2005). It was hoped that this form of gene therapy would have effects on the myocardial vasculature but the results were inconclusive. The VEGF gene transfer did not significantly improve stress-induced myocardial perfusion abnormalities compared with placebo plasmid. However, improved regional ventricular wall motion, as assessed by ventriculography, may indicate a favorable anti-ischemic effect (Kastrup *et al*., 2005). Direct injection of rhVEGF into intramycardial tissues would be ineffective as rhVEGF has a very short half life, of between three and six minutes *in vivo* (Li *et al*., 1995; George *et al*., 2000; Pantely and Porter, 2000). This timeframe plus cardiac perfusion would impact on the angiogenic effects of direct rhVEGF injection. A recent review discussing VEGFs applications in clinical medicine showed that promising pre-clinic results obtained in animal experiments with VEGF therapy have not yet been translated into clinical success. VEGFs have tremendous potential as vascular therapeutics and ongoing developments in gene delivery techniques are expected to lead to the generation of novel treatment for ischemic cardiovascular disease (Yla-Herttuala *et al*., 2007).

Of all the factors studied VEGF appears to be one of the most potent inducers of

angiogenesis. Much research has been carried out on VEGF over the last two decades to the point that we now can see a future where VEGF in many of its forms may be used to treat human injuries.

Placental Growth Factor

PlGF, a member of the VEGF superfamily is highly expressed in placenta at all stages of human gestation and loss of PlGF impairs angiogenesis (Maglione *et al*., 1991). PlGF binds with VEGFR-1 but not VEGFR-2. Alternative splicing of human PlGF gene generates 4 isoforms, PlGF-1, PlGF-2, PlGF -3, PlGF-4 (Takahashi and Shibuya, 2005).

Basic Fibroblast Growth Factor

Fibroblast growth factor-2 (previously known as bFGF), is a prototypic and extensively studied member of a large family (FGF-1 to 23) of heparin binding, mitogenic growth factors. FGFs show developmental, tissue, cell-specific regulation and they are potent mitogens for cells of mesodermal and neuroectodermal origin and are powerful angiogenic agents (Ornitz, 2000). FGF-2 also has roles in tissue regeneration, wound healing, and tumor progression (Gospodarowicz, 1990). FGF-2 is predominantly found in the normal myocardium and its expression is increased by hypoxia or hemodynamic stress and chronic ischemia (Schaffer and Nanny, 1996).

FGF-2 actions are mediated by its two isoforms, with differing molecular weights. To date, the vast majority of cardiac medicine clinical trials have involved the lo-FGF-2 (low molecular weight) isoform. FGFs bind to plasma membrane tyrosine kinase receptors, called

FGFRs (Kardami, 2007). It is believed that lo-FGF-2 is localized in the extracellular space, the cytosol of cells and it translocates to the nucleus during the G1-S transition of the cell cycle (Bossard *et al*., 2003). During cell injury and repair FGF-2 is involved in repair, tissue regeneration (Detillieux *et al*., 2004). As this process requires angiogenesis, FGF-2 has a vital regenerative function in these events. FGF-2 functions by receptor binding (Ornitz, 2000). Vessels generated by VEGF tend to be "capillary like and leaky" but those produced by FGF-2 appear to be more mature (Abo-Auda and Benza, 2003). FGF-2 affects smooth muscle cells, fibroblasts, and ECs (Slavin, 1995). The ECs are stimulated to produce plasminogen activators and matrix metalloproteinases causing extracellular breakdown and vascular remodeling. In animal studies, increased EC proliferation, increased collateral vessel density, higher perfusion pressure and improved regional blood flow have been noted, showing the ability of FGF-2 to induce angiogenesis (Baffour *et al*., 1992).

C. Angiogenic Growth factors and the Dental Pulp

Growth factors which affect the pulp have two sources. Pulp cells express VEGF in healthy and pathological situations such as irreversible pulpitis (Artese *et al*., 2002). These cells include odontoblasts, fibroblasts, undifferentiated mesenchymal cells, macrophages (found in the inflamed pulp), lymphocytes (B and T), dendritic cells, Schwann cells, pericytes and endothelial cells. But the odontoblasts and undifferentiated pulp cells are the most important cellular sources of VEGF in the dental pulp (Telles *et al*., 2003). High levels of VEGF secreted by unstimulated pulp cells provide a pro-angiogenic input that appears to be necessary to maintain pulp vascularization (Botero *et al*., 2003).

The other source of growth factors is the dentin matrix. The origin of these growth factors in dentin matrix is probably largely the odontoblast cell (Smith *et al*., 1990; Smith, 2003). Dentin Matrix contains a cocktail of biologically active molecules with a wide range of effects once released. Whilst dentin matrix is not generally considered to show appreciable turnover or remodeling, trauma to the tissue might lead to release of these molecules (Roberts-Clark and Smith, 2000) (Table 1). During the carious process, acids released from bacteria diffuse through the dental tissue. They dissolve enamel and dentin matrix causing the release of growth factors. These growth factors are contained in both the soluble and insoluble tissue compartments of the matrix. Their release may vary under different tissue conditions. The sequestration of growth factors within dentin matrix provides a pool of growth factors that can be mobilized in injury. Fibroblasts and other pulp cells may be other sources. Thus, the overall response in the pulp is likely to be the summation of the effects caused by the release of growth factors from the dentin matrix and local secretion from

pulp cells (Smith *et al*., 1990).

Growth factors released from dentin include VEGF (Roberts-Clark and Smith, 2000). FGF-2 PlGF, PDGF-AB and EGF have been also been isolated in dentin (Roberts-Clark and Smith, 2000). Once unconstrained by the dentinal matrix these growth factors may play a role in pulpal angiogenesis. These factors are involved in pulpal healing (Roberts-Clark and Smith, 2000) and are free to bind to their receptive receptors. VEGFR-2 has been identified in young permanent teeth. This receptor, localized by immunohistochemistry, has a uniform distribution in dental pulp tissue (Grando Mattuella *et al*., 2007a). Therefore the release of growth factors could account for the increased local angiogenesis seen at sites of dental tissue repair after carious or traumatic injury (Smith *et al*., 1990).

FGF-2 is also a potent mitogen for human pulp cells and it inhibits the expression of the odontoblast phenotype by the cells at least partly at pre-transcriptional levels (Shiba, 1995). FGF-2 suppresses terminal differentiation and calcification in pulp cell cultures (Shiba, 1995). FGF-2 receptors as well as VEGF receptors have been immunohistochemically localized in the dental pulp. Receptors were also localized in the developing dental pulp, in preameloblasts and the distal ends of differentiating ameloblasts (Cam *et al*., 1992; Tanikawa, 1999). Once a pulp has been traumatized complete healing requires not only reparative dentine production but also angiogenesis and nerve fiber growth. VEGF and FGF-2 have been demonstrated in pulp fibroblasts by immunohistochemistry and their release increased shortly after injury, showing a very rapid response suggesting a role directly linked to injury. These growth factors exert their angiogenic effects as soluble factors. The release of these factors occurs quickly following injury. Odontoblast progenitor cell

migration to the injury site may require newly formed blood vessels. These can be initiated by the secretion of angiogenic growth factors by dental pulp fibroblasts (Tran-Hung *et al*., 2006).

Bacteria products that reach the dental pulp during the carious process or a result of trauma also have a role to play in stimulating pulpal angiogenesis. LPS (Endotoxin) from gram negative bacteria can induce VEGF in pulp cells (Matsushita *et al*., 1999). VEGF protein expression is upregulated in (mouse) odontoblast like cells and macrophages exposed to LPS, but not in undifferentiated pulp cells or fibroblasts. Odontoblasts and macrophages are thus the key players in pulpal neovascularization in teeth with deep carious lesions (Botero *et al*., 2003).

LTA another bacterial product, from gram positive streptococci can induce up regulation in VEGF protein expression in macrophages, odontoblast cells and in undifferentiated pulp cells. LTA does not induce VEGF expression in fibroblasts. VEGF mRNA expression remains constant upon exposure to LTA, suggesting that VEGF upregulation in these cells is post-transcriptional. Therefore LTA from gram positive bacteria might have a direct role in the enhanced neovascularization observed in infected sites of pulp tissue (Telles *et al*., 2003).

The pulp, when irritated by caries or traumatic injury reacts by releasing pro-angiogenic factors. These are supplemented by factors released from carious demineralized dentin. The net result is angiogenesis with resultant regeneration, but only if the injury is minor. As the intensity of the injury increases with resultant infection of the pulp, the regenerative potential of these pro-angiogenic factors will ultimately be overwhelmed.

D. Tooth Slice Based Models for the Study of Human Dental Pulp Angiogenesis

Originally, research using Tooth Slice Models was undertaken not to study pulpal angiogenesis and revascularization but rather to investigate *in vitro* tissue injury processes in the dentin-pulp complex (Magloire *et al*., 1996). Culture models of this nature were useful for testing factors regulating pulpal repair. In one of the first models, cavities were prepared occlusally in extracted caries free, non-restored third molars of 12-22 year old patients to simulate injury of the dentin-pulp complex. Longitudinal tooth slices (crown to apex) of dentin-pulp complex, 0.75 mm thick were cultured *in vitro* in Basal Medium Eagle supplemented with 50 μg/ml ascorbic acid, 50 μg/ml streptomycin, 100 IU/ml penicillin and 10% fetal calf serum. Under the injured zone at day 0, the cytoplasm of cells appeared to have coagulated and there were vacuoles beneath the dentin. The rest of the pulp remained unchanged. At 12 days under the injured dentin, elongated cells were seen aligned along the edge of the pre-dentin and at 16 days, this was more evident. Tissue recovery was observed at the periphery of the pulp. Neovascularization was evident as packed thin vessels parallel to the dentin walls or subjacent to the cell layer underlying the prepared cavities. Sound polarized odontoblasts were observed in cultures maintained for 21 days under healthy non-injured dentin. Long-term maintenance of the odontoblast phenotype was correlated with the presence of dentin matrix components released during demineralization caused by the ascorbic acid in the culture medium. This study demonstrated that severed dentin-pulp complex from human teeth could be cultured *in vitro* (Magliore *et al*., 1996).

Tooth slice models involving rat teeth have also been used to study tissue injury and repair processes in the dentine-pulp complex over long periods of time (Sloan *et al*., 1998). 2 mm

thick slices were cultured for 14 days in DMEM supplemented with 0.15 mg/ml ascorbic acid, 10% heat inactivated fetal calf serum, 200 mM L-glutamine, 1% penicillin/streptomycin and 1% low melting agar. After 2 days of culture, it was noted that cell and tissue morphology was preserved throughout the dentine-pulp complex. Odontoblasts were visible as tall, columnar cells with polarized basal nuclei. They were in contact with the pre-dentin and the dentine extracellular matrix. Fibroblasts of the central core of the pulp were spindle-shaped and of similar density to those of the uncultured tissues. The morphological appearance resembled that of uncultured tissues. After 5 days, odontoblasts had maintained their phenotypic morphology and were in contact with the dentin extracellular matrix. Normal tissue morphology was still evident in cultures of 7 days. At 14 days the polarity and the morphology of the odontoblasts and the integrity of the dentine pulp complex was well maintained. However, after 14 days the odontoblasts began to show some ultrastructural changes. There was a reduction in the length of the cell bodies. Their columnar appearance became less distinct. This model maintained the vitality of the dentin pulp complex up to 14 days. It was demonstrated that this model could be used to study cell-matrix interactions in mature dental tissues and regulatory processes controlling cell activity in the dentine-pulp complex. This was the first indication that angiogenesis could possibly be studied in severed dental pulps, but only up to 14 days *in vitro* (Sloan *et al*., 1998).

Further studies cultured 2 mm thick; rat incisor tooth slices for 10 days (Murray *et al*., 2000) or 21 days (Saw *et al*., 2005). These pulp-dentin complexes were cultured *in vitro* with a view to analyzing the effect of dental materials on the pulp. These involved either direct or indirect contact of the materials with the dentin-pulp complex (Saw *et al*., 2005).

Throughout the culture period, no morphological alterations were seen in dentin-pulp complex. The pulp tissue, contained in the tooth slices remained viable over a 10 day experimental period (Murray *et al*., 2000). There were no changes in the cell densities of odontoblasts and fibroblasts in the central pulp core after 7 days in culture, but after 14 days, pulpal cells within the tooth slice could not maintain their cell densities and normal morphological changes. Odontoblasts appeared shrunken with loss of cellular details and fewer pulp fibroblasts were identified. There was a dramatic decrease of viable cells after 21 days in culture (Saw *et al*., 2005). From these models, it appears that between 7 and 14 day *in vitro* culture is viable but 7 days is optimal.

Recently it was shown that VEGF is capable of inducing an angiogenic response in dental pulps *in vitro* (Gonçalves *et al*., 2007). A tooth-slice based *in vivo* model system was developed to study the effect of VEGF on angiogenesis and revascularization events in severed human dental pulp. It was shown that this might become a useful model for studies of novel therapeutic strategies for the treatment of avulsed teeth (Gonçalves *et al*., 2007). The Tooth Slice Based Model utilized was based on Anthony Smith's original *in vitro* approach for the study of dentinogenesis by organ culture of the dentin-pulp complex from rat incisor teeth (Sloan *et al*., 1998). Non-carious human third molars were sliced with a sterile diamond saw cross-sectionally at the CEJ to produce 1.5 mm thick slices. For the *in vitro* part of the study tooth slices were cultured in tooth slice culture media (High glucose DMEM supplemented with 20% heat inactivated fetal bovine serum (FBS), 5 ml L-glutamine 200 mM, 5 ml of 10,000 units of penicillin and 10 mg/ml streptomycin, 1 ml Amphotericin B 1.25 mg/ml and 0.15 mg/ml vitamin C) supplemented with 50 ng/mL $rhVEGF_{165}$. Tooth slices were cultured for seven days. The pulp tissues retained the overall

histological features of normal dental pulps, having intact odontoblast layers. Adding VEGF to the culture media resulted in an increase in microvessel density compared with untreated controls (Gonçalves *et al*., 2007).

The *in vivo* protocol used in the study involved implanting tooth slices in the subcutaneous tissues of SCID mice for 7 days. The dental pulp tissue of all tooth slices implanted in the mice remained vital and an intact odontoblast layer was maintained in the tooth slices for the duration of the experiment (Gonçalves *et al*., 2007). The dental pulps were highly vascularized at the time of implant retrieval. The presence of functional (blood carrying) blood vessels in the dental pulps of the tooth slices implanted suggest that the existing blood vessels from the severed dental pulp were capable of anastomizing with host blood vessels. This might have been accomplished by hypoxia generated locally prior to implantation which stimulated the expression of VEGF, presumably through the activation of hypoxia inducible factor and initiated angiogenic cascades in the pulp tissue. Another possible mechanism is during the tooth slicing process growth factors including VEGF were released from the dentin matrix and directly stimulated angiogenesis and revascularization of the severed pulp tissue.

Presently it is possible to study revascularization events in the dental pulp *in vivo*. The current model was achieved by advances in the characterization of *in vitro* tooth slice models. These were initially used to study the regenerative events of dental pulps in response to injury, the response of pulps to dental restorative materials and pulpal angiogenesis in severed human dental pulps, treated with VEGF *in vitro*.

SECTION II

1. ABSTRACT

Tooth avulsion is a common type of trauma in children and young adults. Replantation is the treatment of choice. Complications such as ankylosis, pulpal necrosis, pulpal obliteration, external root resorption make the long-term prognosis of avulsed teeth unpredictable. Recently, it was shown that vascular endothelial growth factor (VEGF) is capable of inducing an angiogenic response in dental pulps *in vitro*. However, we do not know if this angiogenic factor induces revascularization of severed dental pulps *in vivo*. The purpose of this study was to evaluate the effect of VEGF on the revascularization of human pulp tissue of tooth slices implanted subcutaneously in severe combined immunodeficient (SCID) mice. Non-carious human third molars were sliced with a sterile diamond saw (Model 650, Southbay Technology) cross-sectionally at the CEJ to produce 1 mm thick slices. Slices were treated *ex vivo* in culture media (DMEM) supplemented with 50 ng/ml $rhVEGF_{165}$ or in control culture medium. After 1 hour, tooth slices were implanted subcutaneously in SCID mice. After 14 days, mice were euthanized, tooth slices retrieved, and dental pulp microvessel density was quantified by counting the number of Factor VIII positive blood vessels in four high powered fields per pulp slice (12 tooth slices per condition). Independent experiments were carried out to verify data reproducibility, and statistical analyses (Kruskal-Wallis One Way Analysis of Variance on Ranks and Tukey's Test) were performed using Sigmastat 2.0 software. We observed that the human dental pulps of the tooth slices remained viable for 14 days subcutaneously in the SCID mice under this experimental protocol. Notably, pre-treatment with VEGF prior to implantation increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls. We conclude that VEGF enhances neovascularization of severed human dental pulps *in vivo*. These results may have implications on the treatment of avulsed immature teeth *ex vivo*, prior to replantation.

2. INTRODUCTION

Trauma to the oral region is frequent in children and adolescents. It comprises 5% of all injuries for which treatment is sought, and in preschool children these traumatic injuries can be as high as 18% of all injuries. Of all facial injuries, dental are the most common. Avulsions occur in 1–16% of all dental injuries (Andreasen *et al*., 2007). The avulsion of permanent teeth is the most serious of all dental injuries. The prognosis depends on measures taken at the place of accident or immediately after the avulsion. Replantation is the treatment of choice, but cannot always be carried out immediately (Flores *et al*., 2007b). If this can be achieved pulpal revascularization could be expected in 41-93% of cases (Andreasen *et al*., 1995b). Revascularization is rare in mature replanted teeth of children and adolescents with closed apices (Cvek *et al*., 1990). If immediate replantation cannot be carrier out complications can occur. These include ankylosis, pulpal necrosis, pulpal obliteration, external root resorption (Andreasen *et al*., 1995a, 1995b). The most successful outcome of replantation is pulpal healing, but if this is to occur, revascularization of the severed pulpal vasculature must take place (Strobl *et al*., 2003). This is difficult especially in cases of delayed replantation (Ebeleseder *et al*., 1998).

Angiogenesis is defined as the process of developing new blood vessels from pre-existing capillaries (Folkman and Shing, 1992). It is an important part of many natural processes, such as embryonic development and ovulation wound repair. It is also involved in pathological processes such as arthritis, diabetic retinopathy, and tumor growth. In many diseases the body looses control of angiogenesis resulting in excessive blood vessel development as observed in cancer (Folkman and Shing, 1992). Vascular Endothelial growth factor (VEGF) plays a central role in angiogenesis, promoting the formation of new capillaries. VEGF increases

vascular permeability (Dvorak *et al*., 1985; Dvorak *et al*., 1995). Basic Fibroblast Growth Factor (FGF-2) is another potent angiogenic factor that shows increased expression in hypoxic conditions (Kardami, 2007). FGF-2 causes increased endothelial cell proliferation and collateral vessel density (Baffour *et al*., 1992).

VEGF has received much attention regarding its potential therapeutic use in therapeutic angiogenesis for the treatment of vascular conditions such as myocardial ischemia (Kastrup *et al*., 2005; Yla-Herttuala *et al*., 2007). Promising preclinical results obtained in animal experiments with VEGF therapy have not yet been translated into clinical success (Yla-Herttuala *et al*., 2007). VEGF has tremendous potential in vascular therapeutics and with further developments in gene delivery techniques it may be possible to develop a novel treatment for ischemic cardiovascular disease (Yla-Herttuala *et al*., 2007).

Recently, a tooth-slice based *in vivo* model system was developed and characterized for the study of revascularization in the dental pulp. It was shown that VEGF induced angiogenesis in the dental pulps of human tooth slices *in vitro* (Gonçalves *et al*., 2007). However, we do not know if VEGF induces revascularization of severed dental pulps *in vivo*. Using the Tooth Slice Based Model, we explored the application of angiogenic growth factor/s to severed human dental pulps both *in vitro* and *in vivo*, with the implantation of 1 mm thick tooth slices into the dorsal subcutaneous tissue of SCID mice for a two week period. Here we present the results of these experiments. We believe that these results may have implications for the direction of future studies involving the treatment of avulsed immature teeth *ex vivo*, prior to replantation.

3. MATERIALS AND METHODS

***In vitro* Culture of Severed Human Dental Pulps** (Fig. 3)

Vital, non-carious and non-restored human third molars from patients no older than 30 years of age were collected from the Oral Surgery Department of the University of Michigan School of Dentistry, Ann Arbor, Michigan. After extraction, the teeth were immediately placed into sterile Transport Medium i.e. high glucose DMEM (Sigma Chemical Co.) supplemented with L-glutamine (Gibco), penicillin, streptomycin (Gibco) and Amphotericin B (Sigma). The teeth were surface disinfected using a sterile gauze swab soaked in 70% ethanol solution. Excess soft tissue covering the root surface was removed with a sterile curette. Tissue surrounding the apices of the third molars was left intact.

Preparation of Specimens

Teeth were fixed to a wooden block (3 cm x 3 cm x 1.5 cm) by self curing acrylic (Coldpac, tooth acrylic, Moltoid, Chicago, IL) and these blocks were attached to an Isomet Low Speed (Model 650, South Bay Technology, Inc., San Clemente, CA) (Fig. 4) saw mount. The teeth were cut into sections of 1 mm thickness with a lapidary blade 303 Series (MK-303 Professional, MK Diamond Products Inc., Calais, ME) and cooled with sterile PBS-1x-phosphate buffered saline (Gibco). The saw was washed with 70% ethanol and sterile PBS.

Two tooth slices were taken from each third molar. The first tooth slice was cut from the CEJ and the second was taken apically to the first tooth slice. Both slices contained pulp tissue from the pulp chamber. The slices were placed into individual wells of a 12 well plate (BD, Franklin Lakes, NJ). Each well contained enough Culture Media (1ml) (High glucose DMEM

supplemented with 20% heat inactivated fetal bovine serum (FBS) (Gibco), 5ml L-glutamine 200mM, 5ml of 10,000 units of penicillin and 10 mg/ml streptomycin, 1ml Amphotericin B 1.25 mg/ml and 0.15 mg/ml vitamin C (Sigma)) to cover the tooth slice. Sections were then cultured at 37 ^{o}C, in 5 % CO_2 and air, in a humidified (100% H_20) incubator for seven days. The tooth slice culture media was changed the day after sectioning the teeth and every second day thereafter.

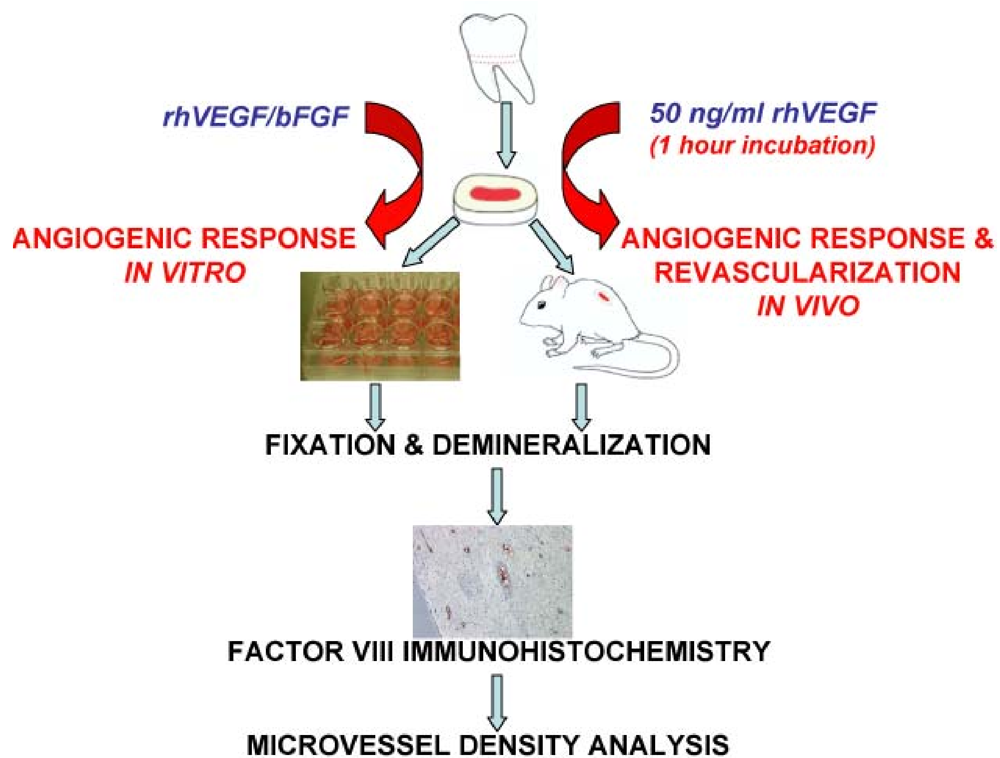

Figure 3. Tooth Slice Model to Study Pulpal Revascularization (Adapted from Gonçalves *et al*., 2007)

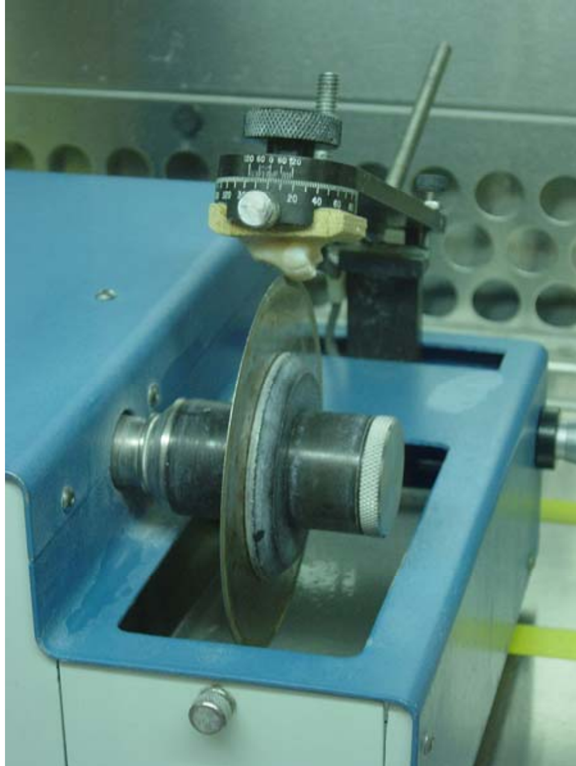

Figure 4. Diamond Wheel Saw Model 650 (Southbay Technologies, San Clemente, California) with a third molar fixed to a wooden mounting block by acrylic resin, in the cutting jig. The sectioning of the third molars was performed in a cell culture hood under aseptic conditions.

Experimental Design

Group 1: four tooth slices cultured in tooth slice culture media (without growth factors; negative control).

Group 2: four tooth slices cultured in tooth slice culture media supplemented with 10 ng/ml rh$VEGF_{165}$ (R&D Systems Inc., Minneapolis, MN).

Group 3: four tooth slices cultured in tooth slice culture media supplemented with 50 ng/ml rh$VEGF_{165}$.

Three independent experiments were performed, using this experimental design. Data was analyzed from 12 slices per experimental condition. A further four independent experiments were also conducted, but the histological sections from all three groups contained areas of pulpal voiding and were unsuitable for microvessel quantification (Fig. 14; page 82 APPENDIX).

The same experimental design was employed for FGF-2, as an alternative pro-angiogenic factor to VEGF. Three independent experiments were performed. Data was analyzed from 12 favorable slices per experimental condition. One independent experiment was also conducted, but the histological sections from all three groups contained areas of pulpal voiding and were unsuitable for microvessel quantification (Fig. 14; page 82 APPENDIX).

Removal of Slices From Culture

After seven days, tooth slices were removed from the media and fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) at 4^oC for 24 hours, followed by demineralization in Decalcifier (Decalcifier II, Surgipath Medical Industries Inc., Richmond, IL) for 22 hours

at RT (APPENDIX). Tissues were processed at the Histology Core University of Michigan, School of Dentistry and embedded in paraffin for histological examination. Three sections of each tooth slice were cut at 5 μm thickness with a microtome and one section was stained with Hematoxylin and Eosin (Sigma Aldrich, St. Louis, MO), two were processed for Factor VIII immunostaining as described (APPENDIX).

In vivo Culture of Severed Human Dental Pulps

Vital, non-carious and non-restored human third molars from patients not older than 30 years of age were collected from the Oral Surgery Department of the University of Michigan School of Dentistry, Ann Arbor, Michigan. After extraction the teeth were placed immediately into sterile Transport Medium. The teeth were handled and sliced as in the *in vitro* experiment (as described previously).

After cutting, the slices were placed into individual wells of a 12 well plate (BD, Franklin Lakes, NJ). Each well contained enough culture medium to cover the tooth slice. The slices were placed into wells which correspond to the correct control and experimental groups. Group 2 tooth slices had the pulpal tissue removed (APPENDIX) prior to placement in the culture media. Group 4 the tooth slices were placed into culture media containing 50 ng/ml $rhVEGF_{165}$. The tooth slices were cultured ex-vivo at 37 ^{o}C, in 5% CO_2 and air, in a humidified incubator for 1 hour prior to implantation.

SCID mice (CB.17 SCID; Charles River, Wilmington, MA) were anesthetized with a solution of ketamine/xylazine. The use and handling of animals in this study was performed in accordance with an approved IRB and UCUCA protocols (APPENDIX). Each SCID mouse

received a 2 cm long incision in the dorsum with a #15 blade scalpel. Bilateral subcutaneous pockets were made upon separation of the dermis from the underlying muscle layer and one tooth slice was placed in each pocket, within 3 hours of extraction. Two tooth slices were implanted into each mouse; a control slice on one side and an experimental slice on the other. The tooth slices were placed in contact with the exposed muscle fibers. The dorsal incision was re-approximated to obtain wound closure using Vetbond Tissue Adhesive (3M Animal Care Products, St Paul, MN). The mice were examined two hours after the surgery for signs of recovery and then every day thereafter for signs of infection at the wound site or other morbidities. The slices remained in the mice for 14 days.

Experimental Design

Group 1: Six slices cultured ex-vivo for one hour and immediately fixed in 10% Buffered Formalin (Fischer Scientific, Pittsburg, PA) after tooth cross-sectional slicing.

Group 2: Six slices implanted for 14 days with no pulpal tissue.

Group 3: Six slices implanted for 14 days. Tooth slices cultured for one hour *ex vivo* with no pro-angiogenic factor added to the tooth slice culture media.

Group 4: Six slices implanted for 14 days. Tooth slices cultured *ex-vivo* for one hour in culture media supplemented with 50 ng/ml $rhVEGF_{165}$.

This experiment was repeated twice to verify reproducibility of results.

Retrieval of the Tooth Slices from the SCID Mice

14 days after implantation mice were euthanized and the slices were removed (Fig. 5; Fig.6). The tooth slices were fixed in 10% buffered formalin (Fisher Scientific) at 4 ^{o}C for 36 hours, followed by demineralization in Decalcifier (Decalcifier II, Surgipath Medical Industries Inc., Richmond, IL) for 22 hours at RT, which were then processed and embedded in paraffin for histological examination (Histology Core University of Michigan, School of Dentistry). Three sections of the tooth slice were cut at 5 μm with a microtome and one section was stained with Hematoxylin and Eosin (Sigma Aldrich), two were processed for Factor VIII immunohistochemistry (APPENDIX).

Figure 5. Retrieval of tooth slices from the SCID mouse,

after 14 days implantation

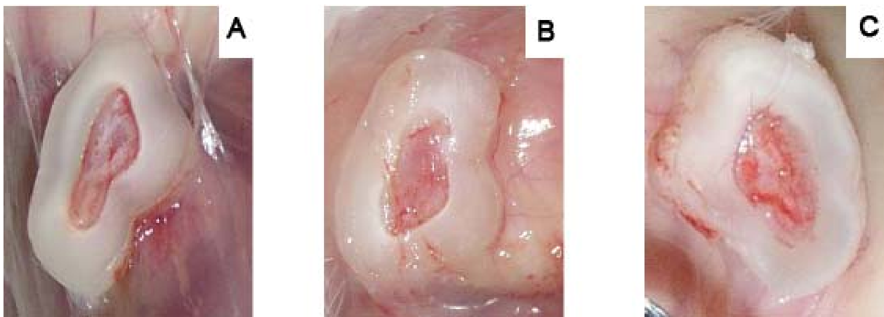

Figure 6. Implants after two weeks. (A) Tooth slice implanted with no pulp tissue. Notice the white bands of mouse connective tissue which invaded the pulp space. (B) Tooth slice with severed human dental pulp cultured for one hour *ex vivo* in tooth slice culture media. (C) Tooth slice with severed human dental pulp cultured for one hour *ex vivo* in tooth slice culture media supplemented with 50 ng/ml of $rhVEGF_{165}$.

Immunohistochemistry

Factor VIII Immunohistochemistry was performed on each tooth slice to quantify blood vessel number per high powered field. Factor VIII Immunohistochemical analysis was performed using Dako CytomationEnVision + System-HRP (AEC) with rabbit primary antibodies. Histological sections were incubated with 1:250 polyclonal rabbit anti-human Factor VIII (Neomarkers, Fremont, CA) over night at 4 ^{o}C. Sections were washed with Wash Buffer (Dako), and incubated with second antibody (Labelled Polymer-HRP, anti-rabbit Dako) for 45 minutes at room temperature. Sections were washed with Dako Wash Buffer and AEC substrate chromogen (Dako) was applied for 2 minutes, the reaction was observed under a light microscope at 200x magnification. Sections were counterstained with hematoxylin. The slides were washed again with distilled water, dried and then a cover slip was placed over the histological section and fixed to the slide with aqueous mounting media (Vecta Mount AQ). Control histological sections were incubated over night at 4°C with 1:250 polyclonal rabbit anti-human IgG (Fig. 8).

Microvessel Quantification

Quantitative analysis of blood vessel densities was performed by ascertaining the number of Factor VIII stained blood vessels in four 200X high powered fields, per tooth slice. A total of 12 tooth slices were examined for each condition. 200x Images were acquired using Image Pro Plus (version 5.1.2.59 for Windows XP, Media Cybernetics Inc., Silver Spring, MD) software and a microscope (Eclipse E800, Nikon, Melville, NY). Four, 200x fields were chosen at random from each tooth slice, using a grid superimposed on a digital image, captured by a Digital Camera (RT Slider, Diagnostic Instruments Inc., Sterling Heights, MI)

(Fig. 7). Each grid box was the same size as a 200x field captured by the camera. Fields were chosen that contained pulp only. If dentin was visible on the periphery of the pulp, then this field was not selected. If more than four fields were available for selection in a row of grid boxes in the center of the pulp, then four boxes were chosen at random (Fig. 7).

Statistical Analysis

Statistical analysis was performed using One Way ANOVA followed by Tukey's Test for multiple group comparison and Kruskal-Wallis One Way Analysis of Variance (for non normally distributed data) on Ranks with SIGMASTAT 2.0 statistical software (SPSS, Chicago, IL). The level of significance was determined at $P \leq 0.05$.

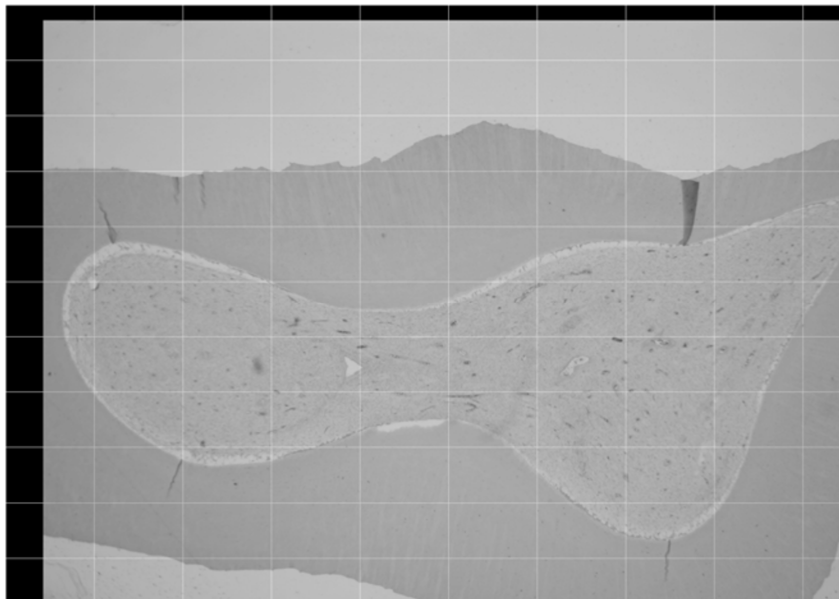

Figure 7. Grid superimposed on a 20x histological image using Image Pros Plus (version 5.1.2.59 for Windows XP, Media Cybernetics Inc, Silver Spring, MD). Each grid rectangle is the same dimension as the 200X field image as visualized with the microscope (Nikon Eclipse E800, Melville, NY).

4. RESULTS

In vitro Effect of VEGF and FGF-2 on Severed Human Dental Pulps

We observed that after culturing the tooth slices *in vitro* for 7 days, the pulp tissues retained the histological features of normal pulps and remained viable under this experimental protocol (Fig. 8 A, B, C, E, F, G).

In order to evaluate the effect of VEGF and FGF-2 on dental pulp angiogenesis, we cultured the tooth slices in the presence or absence of VEGF or FGF-2 and performed immunohistochemical staining to identify blood vessels (Fig. 8 F, G). Adding VEGF resulted in an increase in dental pulp microvessel density ($p<0.05$) compared to untreated controls (Fig. 8 I). FGF-2 treatment of tooth slices *in vitro* also increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls (Fig. 8 I).

In vivo Effect of VEGF on Severed Human Dental Pulps Implanted in the Subcutaneous Tissues of SCID Mice

It has been shown that human blood vessels can be engineered by seeding primary human endothelial cells in scaffolds using the SCID Mouse Model of Human Angiogenesis (Nör *et al*., 2001). These experiments provided the basis for the implantation of tooth slices in the dorsal subcutaneous tissue of SCID mice. To evaluate the effect of VEGF on dental pulp angiogenesis *in vivo*, tooth slices were cultured in the presence or absence of VEGF for 1 hour *ex vivo* prior to 14 day implantation. Immunohistochemical staining was performed to identify blood vessels (Fig. 9 D, E, F). Adding VEGF resulted in an increase in dental pulp microvessel density ($p<0.05$) compared to untreated controls (tooth slices not implanted but

cultured *ex vivo* for 1 hour, implanted with no pulp and untreated implanted pulp slices) (Fig. 9 G). The pulp tissues retained the histological features of normal pulps (Fig. 9 A, B, C) under this experimental protocol and the severed human pulp tissue of all tooth slices implanted remained vital under the experimental protocol (Fig. 9 A-F). The dental pulps were highly vascularized at the time of implant retrieval. Red blood cells were visualized in the lumen of blood vessels stained with the Factor VIII Antibody (Fig. 9 F). This demonstrates that the severed human vasculature had anastomized with the SCID mouse vasculature and allowed the severed human pulp tissue to remain viable during the experimental period.

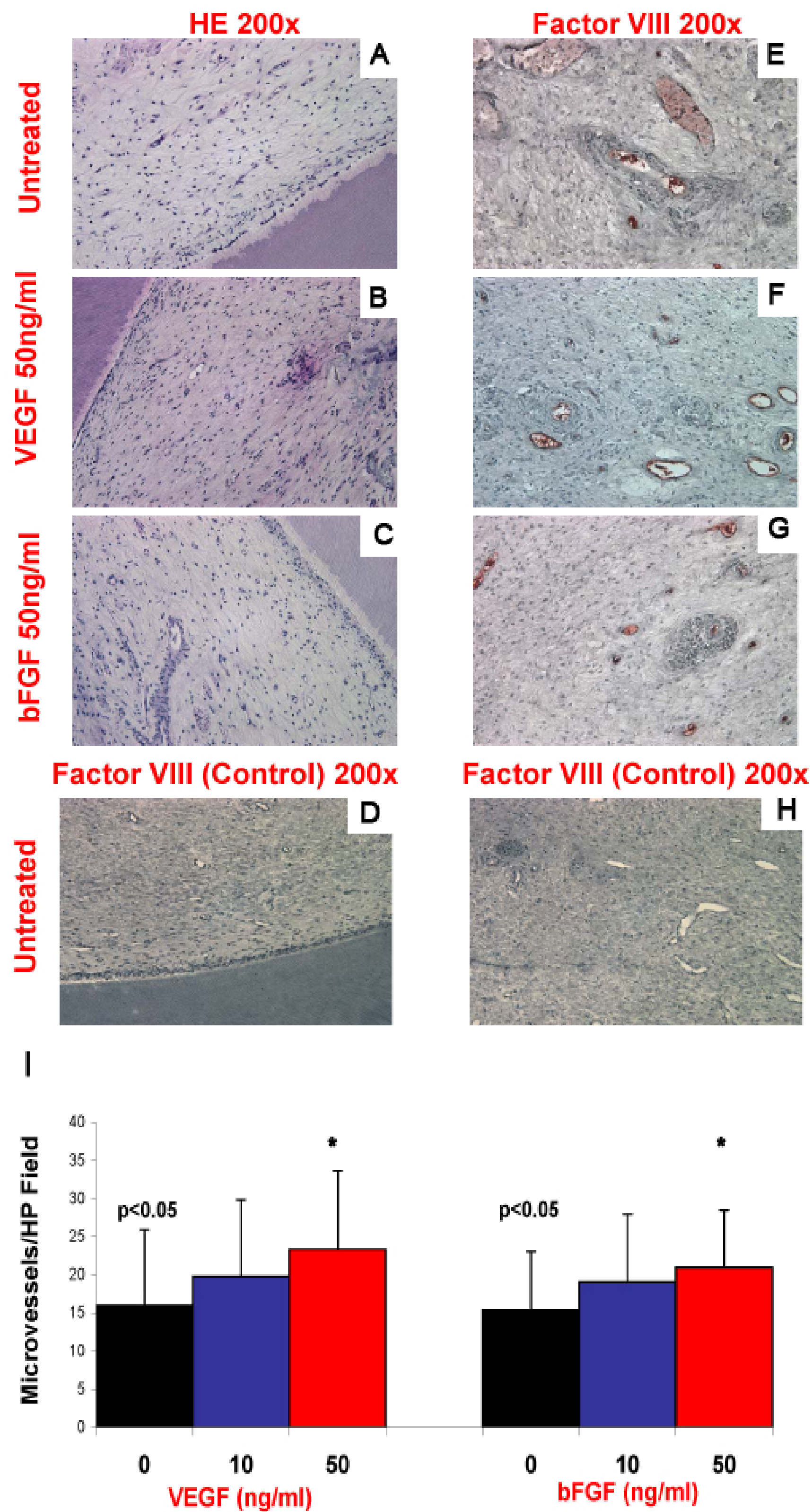

Figure 8. Effect of VEGF on *in vitro* angiogenesis in severed human dental pulp. (A, D, E, H) Tooth slices were untreated or (B, F) cultured in presence of 50ng/ml of $rhVEGF_{165}$ or (C, G) rhFGF-2. (A, B,

C) Hematoxylin/eosin staining of dental pulps after 7 days in culture (200x). (E, F, G) Microscopic fields of Factor VIII Immunohistochemistry for identification of blood vessels (red staining). (D, H) Negative control; primary rabbit anti-human Factor VIII antibody, substituted with rabbit anti-human IgG antibody. Notice no red stained microvessels (D, H). (I) Graph depicting the mean (±SD) number of Factor VIII positive blood vessels from 48 fields per condition. Asterisk indicates statistical significance ($p<0.05$) (Kruskal-Wallis One Way Analysis of Variance on Ranks and Tukey's Test).

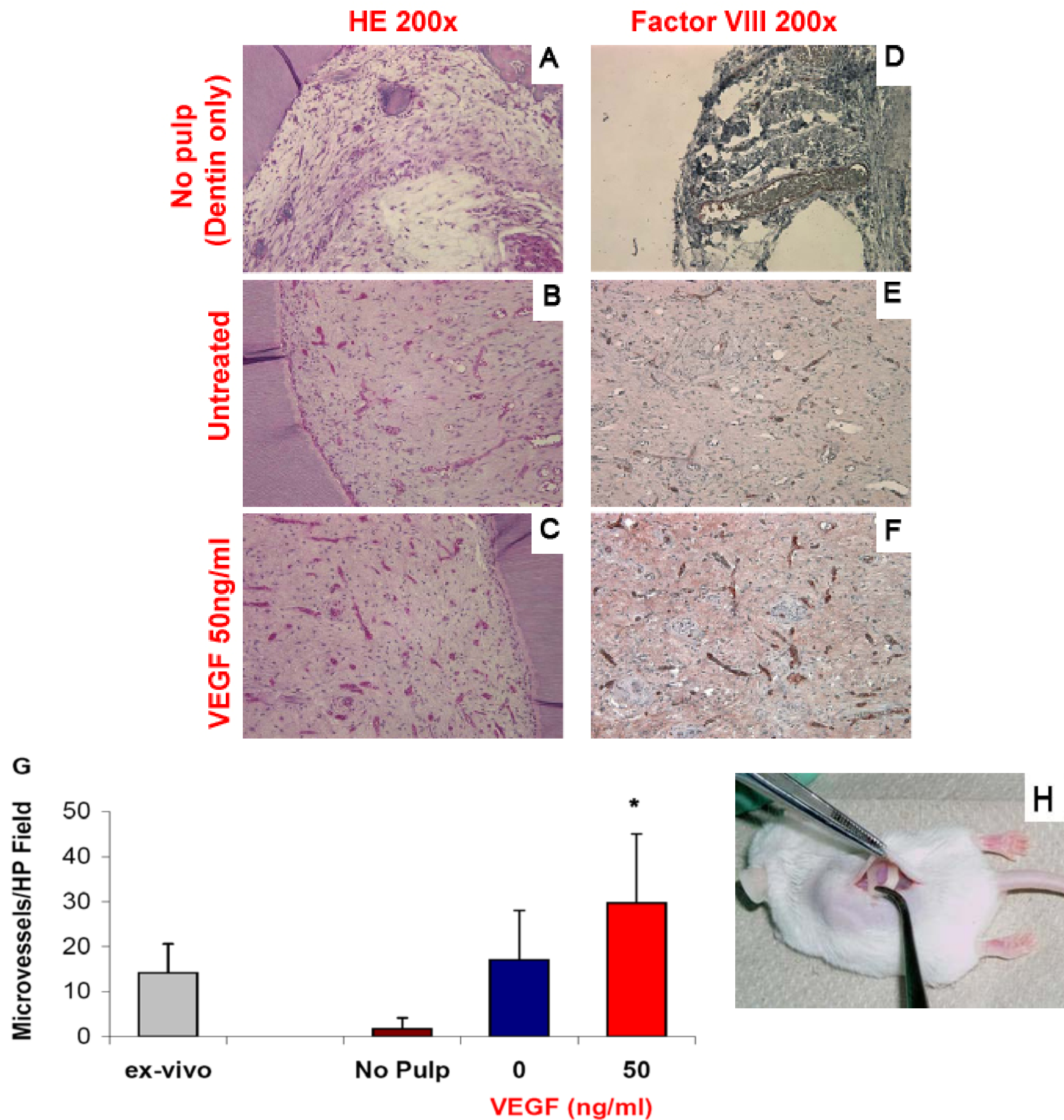

Figure 9. Effect of VEGF on *in vivo* angiogenesis in human dental pulp. (A, D) Tooth slices implanted with no pulp tissue, (B, E) with untreated pulp tissue, (C, F) with pulp tissue treated with 50ng/ml of rhVEGF165 for 1 hour *ex-vivo* prior to implantation. (A, B, C) Hematoxylin/eosin staining of dental pulps after 14 days implantation (200x). (D, E, F) Microscopic fields of Factor VIII Immunohistochemistry for identification of blood vessels (red staining). (G) Graph depicting the mean (±SD) number of Factor VIII positive blood vessels from 48 fields per condition. Asterisk indicates statistical significance ($p<0.05$). (Kruskal-Wallis One Way Analysis of Variance on Ranks and Tukey's Test). (H) Tooth slice implanted into a dorsum pocket of a SCID mouse.

5. DISCUSSION

This study builds on the principles that were developed and initially characterized in a SCID Mouse Model for the study of human angiogenesis (Nör *et al*., 2001; Gonçalves *et al*., 2007). This model was based on tooth slicing culture experiments, first described by Sloan and colleagues (Sloan *et al*., 1998). We demonstrated using a Tooth Slice Model (Fig. 10) that human dental pulps cultured for seven days *in vitro* retained their viability. We also demonstrated that human dental pulps of tooth slices remained viable for 14 days, subcutaneously in SCID mice under this experimental protocol.

Pulp cells release growth factors in response to injury (Botero *et al*., 2006). VEGF is expressed by pulp cells (Telles *et al*., 2003) and is found in dentin matrix (Smith *et al*., 1990; Smith, 2003). It was shown that VEGF is upregulated in odontoblast-like cells that were exposed to bacterial endotoxins (Botero *et al*., 2006). FGF-2 released from human dental pulp following forced tooth movement plays a role in the angiogenic response of the pulp (Derringer *et al*., 2004). The quantity of VEGF and FGF-2 released from pulp cells exposed to bacterial endotoxins is unknown. This is currently under investigation in our laboratory.

$rhVEGF_{165}$ and rhFGF-2 are angiogenic *in vitro* (Nör *et al*., 2002). 50 ng/ml of $rhVEGF_{165}$ has prolongs the survival of human dermal microvascular endothelial cells *in vitro* (Nör and Polverini, 1999; Nör *et al*., 2002). Zero to 50 ng/ml of $rhVEGF_{165}$ were used to examine pro-angiogenic signaling pathways in human dermal microvascular endothelial cells *in vitro* (Karl *et al*., 2005). We used concentrations of 10 ng/ml and 50 ng/ml of $rhVEGF_{165}$ and rhFGF-2 to treat severed human dental pulps *in vitro*. We observed that treatment of tooth slices with 50 ng/ml of $rhVEGF_{165}$ increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls. We also observed that the *in vitro* treatment of tooth slices with 50 ng/ml

of rhFGF-2 increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls. The exogenous $rhVEGF_{165}$ or FGF-2 in the tooth slice culture media, plus the hypoxia induced VEGF in the severed pulp and also the VEGF and FGF-2 released from the dentin matrix during tooth slicing may have provided sufficient pro-angiogenic stimuli to enhance tissue microvessel density.

Angiogenesis occurs at pre-existing capillaries (Folkman and Shing, 1992) and the subodontoblast layer contains a rich vascular network (Takahashi, 1985). Angiogenesis may have occurred in this region to a greater extent compared to the remainder of the pulp. We did not quantify microvessels in the subodontoblast layer. This is a limitation of our study.

We cultured severed human dental pulp tissue *ex vivo* in tooth slice culture media supplemented with $rhVEGF_{165}$ prior to SCID mouse implantation because recent clinical trials involving direct intramyocardial plasmid $VEGF_{165}$ gene therapy in patients with angina pectoris were conducted (Kastrup *et al*., 2005). Results showed that gene therapy did not improve myocardial perfusion but anti-ischemic effects were noted. These trials highlighted the potential that VEGF has to induce tissue revascularization. We treated severed human dental pulp tissues *ex vivo* with 50 ng/ml of $rhVEGF_{165}$ due to our observation that $rhVEGF_{165}$ treatment of tooth slices *in vitro* increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls. We chose one hour *ex vivo* treatment based on the half-life of VEGF. VEGF has a half-life between three and six minutes *in vivo* (George *et al*., 2000; Pantely and Porter, 2000) and approximately one hour *in vitro* (Shi *et al*., 2001; Liu *et al*., 2002).

We used Factor VIII immunohistochemistry to indentify microvessels in severed human dental pulps. Factor VIII immunohistochemistry is widely used in cancer research to identify

vascular “hot spots” in tumors (Uzzan *et al*., 2004; Des Guetz *et al*., 2006). We showed that the polyclonal rabbit anti-human Factor VIII antibody cross reacted with mouse vasculature (Fig. 9D). We obtained tooth slice histological sections which were cut through the severed human pulp tissue and not the mouse connective tissue attached to the exterior surface of the tooth slice. This avoided quantification of mouse vasculature, which would cause erroneous microvessel density analysis of the severed human dental pulps.

Microvessel density analysis is frequently conducted in cancer research and performed by acquiring digital images from 200x high-powered fields of histologically processed tumor tissue (Kim *et al*., 2005; Saravanamuthu *et al*., 2003; Urquidi *et al*., 2002). We acquired 200x high-powered images from each severed human pulp (Gonçalves *et al*., 2007). Image Pro Plus, image analysis software has also been used in microvessel density analysis (Luo *et al*., 2001; Fukasawa and Korc, 2004). We conducted microvessel density analysis using Image Pro Plus and we superimposed a grid (Hochberg *et al*., 2002; Inan *et al*., 2003; Kehrl *et al*., 2004; Trojan *et al*., 2004) onto digital images of each pulp. Microvessels with and without lumens (Weidner, 1995) were counted from chosen fields, using the manual tagging function in the Image Pro Plus software. We chose four random fields from each pulp based on the results of a pilot study, which involved the application of a grid to a random sample of 60, 20x digital images of tooth slices. We determined that four was the minimum number of fields which could be captured per tooth slice, upon grid application.

We observed that the odontoblast layer was maintained in the tooth slices throughout the duration of the experiment and this correlated with the presence of dentin and dentin matrix components (Heywood and Apppleton, 1984; Begue-Kirn, 1992; Sloan *et al*., 1998). Histologically there was evidence of revascularization of the severed human dental pulps

implanted in the subcutaneous tissue of SCID mice. An intact, highly vascularized odontoblast layer was observed (Fig. 9 B, C). The presence of functional blood vessels both in the VEGF treated and untreated tooth slices suggests that mouse blood vessels anastomized with the severed human dental pulp vessels (Fig. 6). This finding agrees with a recent study from our laboratory (Gonçalves *et al.*, 2007). The dental pulp in response to hypoxia may have produced local VEGF, which encouraged revascularization of the dental pulp. Alternatively, growth factors may have been released from the dentin matrix during tooth slicing, triggering local pulpal angiogenesis which influenced the anastomosis of the mouse and human microvessels.

The exogenous $rhVEGF_{165}$ in the tooth slice culture media may have provided sufficient pro-angiogenic stimuli to enhance tissue microvessel density, together with the hypoxia induced VEGF and the VEGF released from the dentin matrix. These events would ultimately result in pro-angiogenic effects, in the severed human dental pulps. We observed that $rhVEGF_{165}$ treatment of tooth slices *ex vivo* prior to implantation in SCID mice increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls.

Our results suggest that $rhVEGF_{165}$ could be used to treat dental pulp conditions that require revascularization. This would include the avulsed immature tooth. At present pulpal revascularization can be expected in 41-93% of cases if replantation is immediate (Andreasen *et al.*, 1995b). Immediate replantation is not always possible. We may in the future be able to increase the chances of revascularization and allow the replanted tooth to have the prospect of becoming more than a space maintainer and a short term solution. Slow release delivery of $rhVEGF_{165}$ in a bioabsorbable polymer would allow application of the angiogenic factor directly to the pulp tissue at the apex of an avulsed immature tooth. A slow release method of

minocycline delivery in the form of Arestin™ microspheres (Ritter *et al*., 2004) could also be applied *ex vivo* in combination with $rhVEGF_{165}$. This would provide both an antimicrobial and pro-angiogenic effect on the severed human pulp tissue. This method of delivering a combination of $rhVEGF_{165}$ and minocycline is currently not available and warrants further investigation. This is truly an interesting prospect but one must remember that this research is in its infancy and is years from human clinical trials.

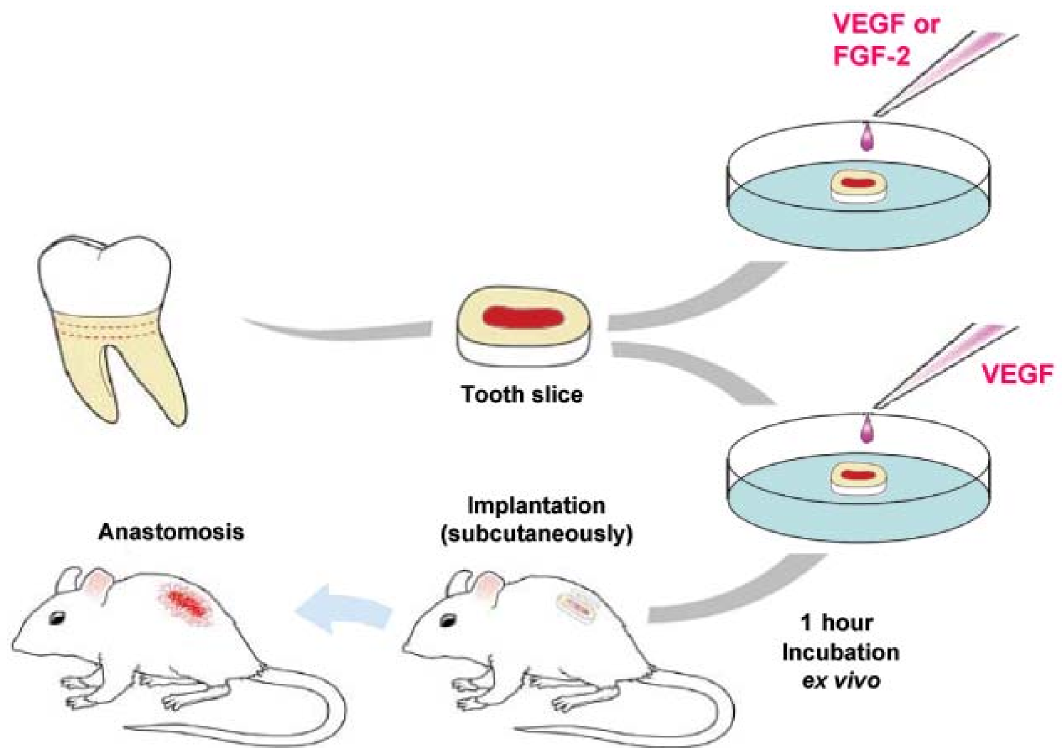

Figure 10. Tooth Slice Model to Study Revascularization of Severed Human Dental Pulp.

6. CONCLUSIONS

The following are the conclusions from our work:

1. rh$VEGF_{165}$ induced angiogenesis in severed human dental pulps of tooth slices treated *ex vivo* prior to implantation in SCID mice. rh$VEGF_{165}$ treatment increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls. Human dental pulps remained viable for 14 days, subcutaneously in the SCID mice under this experimental protocol.

2. We also observed that tooth slices cultured for 7 days remained viable. rh$VEGF_{165}$ and rhFGF-2 treatment induced an angiogenic response in dental pulps of tooth slices *in vitro* and increased the dental pulp microvessel density ($p<0.05$) compared to untreated controls.

3. Results from this study may have implications for the direction of future studies on the treatment of avulsed immature teeth *ex vivo*, prior to replantation. They may also be useful in appreciating the biology involved in human dental pulp revascularization.

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APPENDIX

1. *In vitro* Pilot Study. Three and Seven Day Culture of Severed Human Dental Pulps.

A. Materials and Methods

Part I: Tooth Slice Culture Media not supplemented with $rhVEGF_{165}$

Vital, non-carious and non-restored human third molars from patients not older than 30 years of age were collected from the Oral Surgery Department of the University of Michigan School of Dentistry, Ann Arbor, Michigan. After extraction the teeth were immediately placed into sterile Transport Medium (High glucose DMEM (Sigma Chemical Co.) supplemented with 5 ml L-glutamine (Gibco) 200 mM, 5 ml of 10,000 units of penicillin and 10 mg/ml streptomycin (Gibco) and 1ml Amphotericin B (Sigma) 1.25 mg/l).

The teeth were surface disinfected using a sterile gauze swab soaked in 70% ethanol solution. Excess soft tissue covering the root surface was removed by trimming with a sterile curette. Tissue surrounding the apices of the third molars was left intact.

Preparation of specimens

Teeth were fixed to a wooden block (3 cm x 3 cm x 1.5 cm) by self curing acrylic (Coldpac, tooth acrylic, Moltoid, Chicago, IL ref 44100) and these blocks were attached to an Isomet Low Speed (Model 650, South Bay Technology, Inc.) saw mount. The teeth were cut into sections of 1 mm thickness with a lapidary blade 303 Series (Mk-303 Professional, Mk Diamond Products Inc.) cooled with sterile PBS-1x-phosphate buffered saline (Gibco ref. 10010). The saw was washed with 70% ethanol and sterile PBS. After cutting the slices were placed into individual wells of a multi-well plate (12 well). Each contained enough Tooth

Slice Culture Media (1 ml) (High glucose DMEM supplemented with 20% heat inactivated fetal bovine serum (FBS) (Gibco), 5 ml L-glutamine 200 mM, 5 ml of 10,000 units of penicillin and 10 mg/ml streptomycin, 1 ml Amphotericin B 1.25 mg/ml and 0.15 mg/ml vitamin C (Sigma)) to cover the tooth slice.

Organ culture of the dentin-pulp complex

Tooth slices in group one were slices taken from the CEJ (Large Pulps) and those in group two were sliced from or close to the furcal area of the human third molars (Small Pulps). Small Pulps were cultured for three days, while the Large Pulps were cultured for three and seven days.

Tooth slices were then cultured at $37^{o}C$, in an atmosphere of 5% CO_2 and air, in a humidified (100% H_20) incubator for three and seven days. The tooth slice culture media was changed the day after sectioning and every second day thereafter.

Experimental Design

Group 1: Five Large Pulps (tooth slices) placed in tooth slice culture media for seven days.

Group 2: Five Small Pulps (tooth slices) placed in tooth slice culture media for seven days.

Group 3: Five Large Pulps placed in tooth slice culture media for three days.

Two independent experiments were conducted.

Part II: Tooth Slice Culture Media supplemented with 50 ng/ml rhVEGF$_{165}$

Vital, non-carious and non-restored human third molars from patients not older than 30 years of age were collected from the Oral Surgery Department of the University of Michigan School of Dentistry, Ann Arbor, Michigan and were placed in Transport Media immediately after extraction. The remainder of the protocol is similar to Part I, apart from differences in the experimental design.

Experimental Design

Group 1: Five tooth slices placed in tooth slice culture media for three days.

Group 2: Five tooth slices placed in tooth slice culture media, supplemented with 50 ng/ml of rhVEGF$_{165}$ and cultured for three days.

Group 3: Five tooth slices placed in tooth slice culture media for seven days.

Group 4: Five tooth slices placed in tooth slice culture media, supplemented with 50 ng/ml of rhVEGF$_{165}$ and cultured for seven days.

One independent experiment was conducted.

Removal of slices from Culture

After three or seven days the tooth slices were removed from the culture media and fixed in 10% neutral-buffered formalin (Fisher Scientific ref. SF100-4) at 4°C for 24 hours, followed by demineralization in Decalcifier (Decalcifier II, Surgipath, ref 00460) for 22 hours at room temperature, which were then processed (Histology Core University of Michigan, School of

Dentistry) and embedded in paraffin wax for histological examination. Three sections of each tooth slice were cut at 5µm thickness with a microtome. One section was stained with Hematoxylin and Eosin (Sigma Aldrich, ref. GHS216); two were processed for Factor VIII immunohistochemistry (APPENDIX).

Immunohistochemistry

After retrieval of tooth slices from the culture media after three or seven days and subsequent histological processing, FVIII Immunohistochemistry was performed on each tooth slice to quantify blood vessel number per high powered field. Factor VIII Immunohistochemical analysis was performed using Dako CytomationEnVision + System-HRP (AEC) with Rabbit Primary Antibodies. Histological sections were incubated with (Factor VIII related antigen Ab-1 Rabbit PAb ref. RB-281-A; antibody diluent- ref. S0809, 1:250 polyclonal rabbit anti-human Factor VIII, over night at 4°C. Sections were washed with Wash Buffer (Dako ref. S3006), and incubated with second antibody (Labelled Polymer-HRP, anti-rabbit Dako, ref. 4008) for 45 min at room temperature. Sections were washed with Dako Wash Buffer and AEC substrate chromogen (Dako ref. K4008) was applied for 2 minutes, the reaction was observed under a light microscope at 200x magnification. Sections were counterstained with hematoxylin, after retarding the chromogenic reaction by placing the histological slides in distilled water. The slides were washed again with distilled water, dried and then a cover slide was placed over the histological section and fixed to the slide with aqueous mounting media (Vecta Mount AQ ref H-5501). Slides were processed which contained histological sections from tooth slices that were fixed immediately post tooth slicing. The histological sections were incubated over night at 4°C with 1:250 polyclonal rabbit anti-human IgG (polyclonal

rabbit anti-human) substituted for Factor VIII related antigen Ab-1 Rabbit PAb as a negative control (Fig. 8).

Microvessel Quantification

Quantitative analysis of the blood vessel densities was performed on pulp tissue that was stained with Rabbit Primary Antibodies, by ascertaining the number of Factor VIII stained blood vessels per 200x high powered field, per tooth slice. Three 200x fields were chosen at random, using a microscope (Axioskop, Zeiss, Germany). One field was chosen in the center of the pulp and the other two close to the odontoblast layer, being careful to exclude dentin from the field.

Statistical Analysis

Statistical analysis was performed using One Way ANOVA followed by Tukey's Test for multiple group comparison and Kruskall-Wallis One Way Analysis of Variance on Ranks (for non normally distributed data spread) with Sigmastat 2.0 statistical software (SPSS, Chicago, IL). The level of significance was determined at $P \leq 0.05$.

Part III: Quantification of VEGF and FGF-2 contained in DMEM and 20% FBS (constituents of Tooth Slice Culture Media) by ELISA

VEGF ELISA and FGF-2 ELISA were conducted to ascertain if there were significant amounts of VEGF and or FGF-2 in formulated Tooth Slice Culture Media prior to supplementation with $rhVEGF_{165}$. (APPENDIX: 2B and 2C. Protocols for VEGF ELISA and FGF-2 ELISA).

B. Results

In vitro Pilot Part I

Small and Large tooth slices were prepared under sterile conditions and were cultured for either three or seven days. Following fixation, demineralization, histological processing and Factor VIII Immunohistochemistry, microvessel quantification was performed under this experimental protocol. Immunohistochemical staining successfully identified microvessels (Fig. 11 A-F).

We observed that after culturing tooth slices *in vitro* for three and seven days the human dental pulps remained viable and the tissues retained the overall histological features of normal human dental pulps, with normal odontoblast layers (Fig. 11 A-F). We also noted that there was a dramatic reduction in pulpal voiding (Fig. 14) in the histologically processed 5 μm thick sections. This was a problem we encountered in the first two non pilot $rhVEGF_{165}$ experiments, which were conducted prior to the first pilot study.

This problem was solved by placing the tooth slices retrieved from the culture media, immediately into 10% Neutral Buffered Formalin, chilled at $4^{o}C$. This dramatically reduced pulpal shrinkage.

Microvessel quantification revealed no significant difference between the groups (Fig. 11 G).

In vitro Pilot Part II

Small and Large tooth slices were prepared under sterile conditions and were cultured for either three or seven days. Groups two and four were cultured in tooth slice culture media supplemented with 50 ng/ml VEGF. Following fixation, demineralization, histological

processing and Factor VIII Immunohistochemistry, microvessel quantification was performed. Immunohistochemical staining successfully identified microvessels (Fig. 12 A-H).

We observed that after culturing tooth slices *in vitro* for three and seven days the human dental pulps remained viable and the tissues retained the overall histological features of normal human dental pulps (Fig. 12 A-H). We also noted that there was a dramatic reduction in pulpal voiding (Fig. 14) in the histologically processed 5 μm thick sections. This was a problem we encountered in the first two non pilot $rhVEGF_{165}$ experiments, which were conducted prior to the first pilot study.

This problem was solved by placing the tooth slices retrieved from the culture media, immediately into 10% Neutral Buffered Formalin, chilled at $4^{o}C$. This dramatically reduced pulpal shrinkage.

Adding VEGF to tooth slices cultured for seven days resulted in an increase in microvessels/high powered field as compared to the untreated controls ($p<0.05$) (Fig. 12 I). We observed that VEGF promoted an angiogenic response in severed human dental pulps, which were cultured for seven days *in vitro*.

***In vitro* Pilot Part III**

Trace amounts of VEGF and FGF-2 were found in DMEM and 20% FBS (constituent parts of Tooth Slice Culture Media), but these amounts were not significant and would not interfere with the $rhVEGF_{165}$ or rhFGF-2, which supplemented the Tooth Slice Culture Media in the *in vitro* and *in vivo* experimental protocol (Fig. 13 A, B).

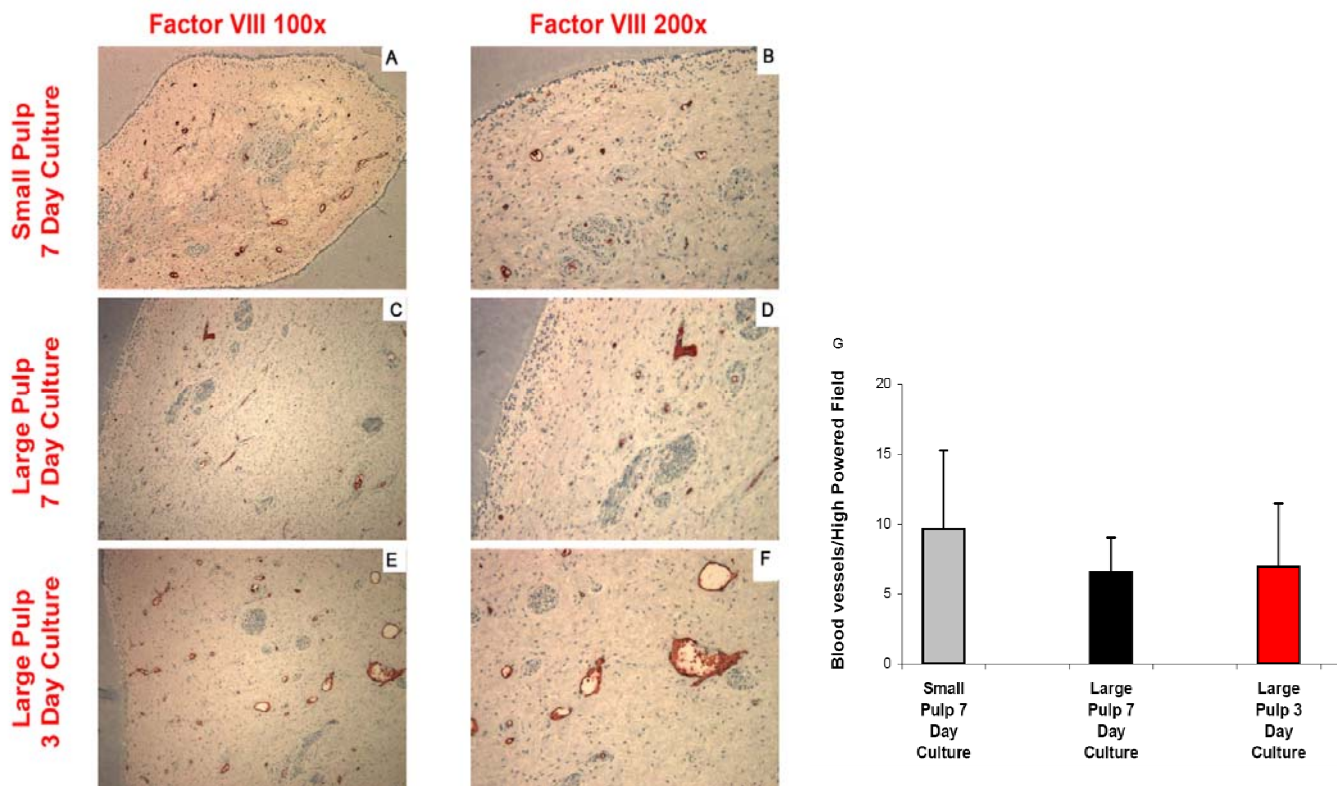

Figure 11. *In vitro* Pilot Study Part I. (A-F) Tooth slices (small and large) were untreated and cultured either for three or seven days. (A-D) Tooth slices cultured for seven days. (E, F) Tooth slices cultured for three days. (A, C, E) Representative microscopic fields of Factor VIII immunohistochemistry for the identification of blood vessels (red staining) (100x) and (B, D, F) (200x). (G) Graph depicts the mean (± SD) number of blood vessels from 15 fields per condition. Graph shows that there is no statistically significant difference between the groups.

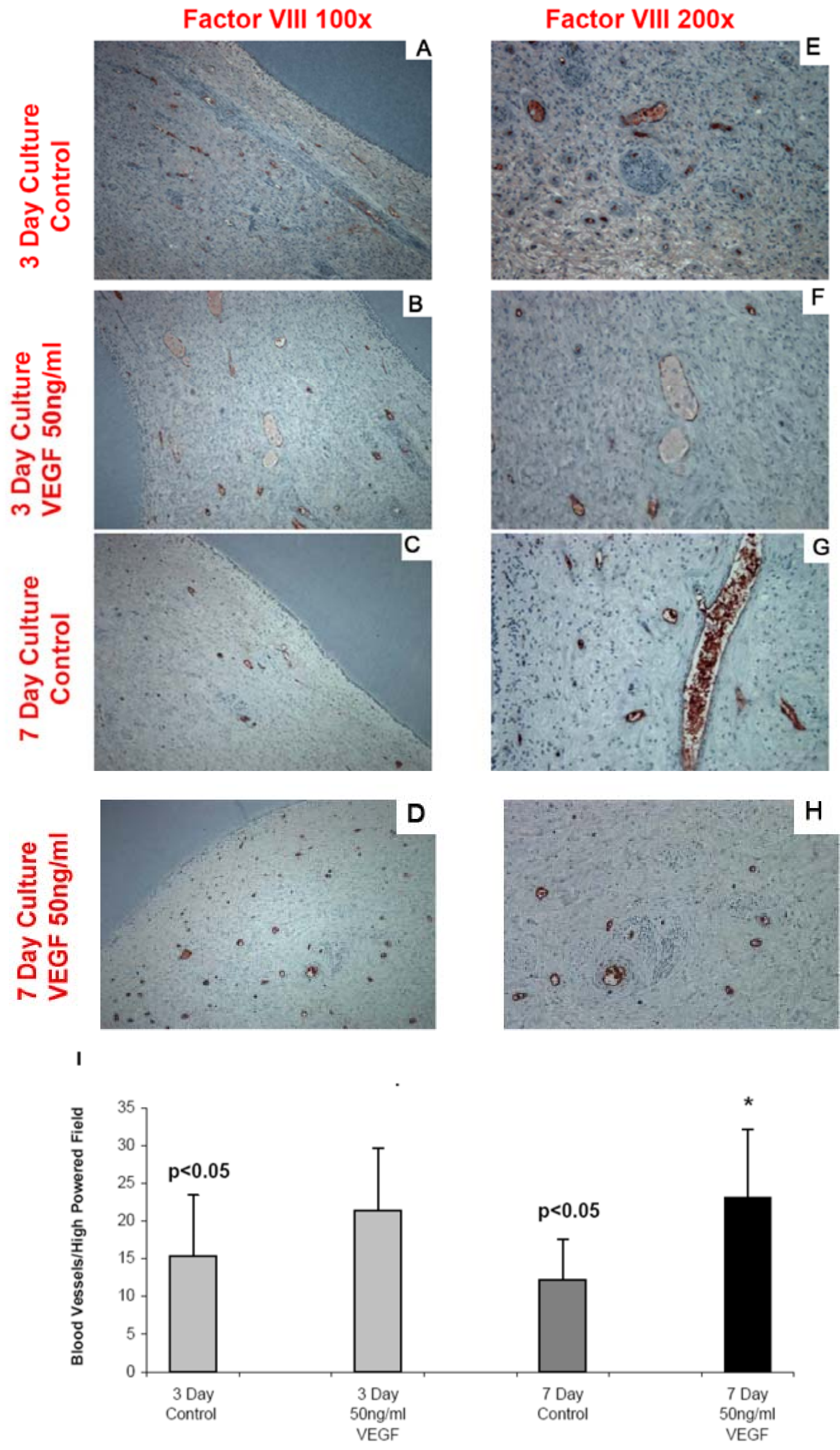

Figure 12. *In vitro* Pilot Study Part II. Effect of VEGF on *in vitro* angiogenesis in human dental pulps. (A-H) Tooth slices (small and large) were either untreated or treated and cultured either for three or seven days. (C, D, G, H) Tooth slices cultured for seven days. (A,

B, E, F) Tooth slices cultured for three days. (A, E, C, G) Tooth slices were untreated or (B, F, D, H) cultured in the presence of 50 ng/ml VEGF. A-D Representative microscopic fields of Factor VIII immunohistochemistry for the identification of blood vessels (100x) and E-H (200x). Note the statistically significant difference between the group treated with 50 ng/ml $rhVEGF_{165}$ for seven days and the two control groups ($p<0.05$).

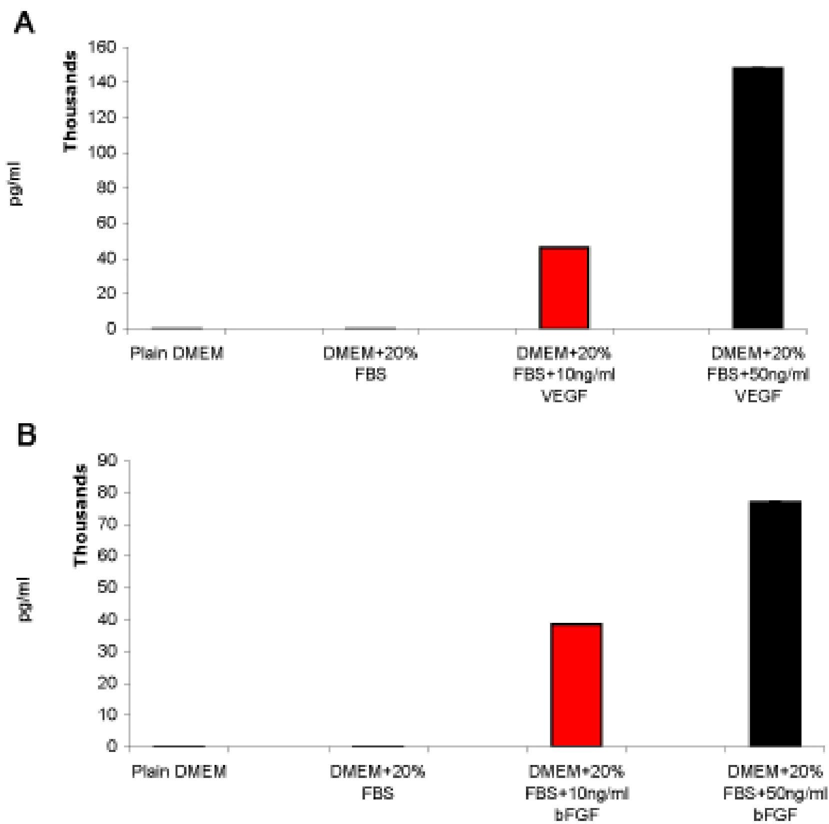

Figure 13. *In vitro* Pilot Part III. Quantification of VEGF and FGF-2 in DMEM and 20% FBS (constituents of Tooth Slice Culture Media) by ELISA. (A) Graph depicting the mean (±SD; not visible as it is a relatively small value) of the pg/ml (Thousands) of VEGF contained in Plain DMEM, DMEM+20% FBS, DMEM+20% FBS+10 ng/ml $rhVEGF_{165}$ and DMEM+20% FBS+50 ng/ml $rhVEGF_{165}$. (B) Graph depicting the mean (±SD; not visible as it is a relatively small value) of the pg/ml (Thousands) of FGF-2 contained in Plain DMEM, DMEM+20% FBS, DMEM+20% FBS+10 ng/ml rhFGF-2 and DMEM+20% FBS+50 ng/ml rhFGF-2.

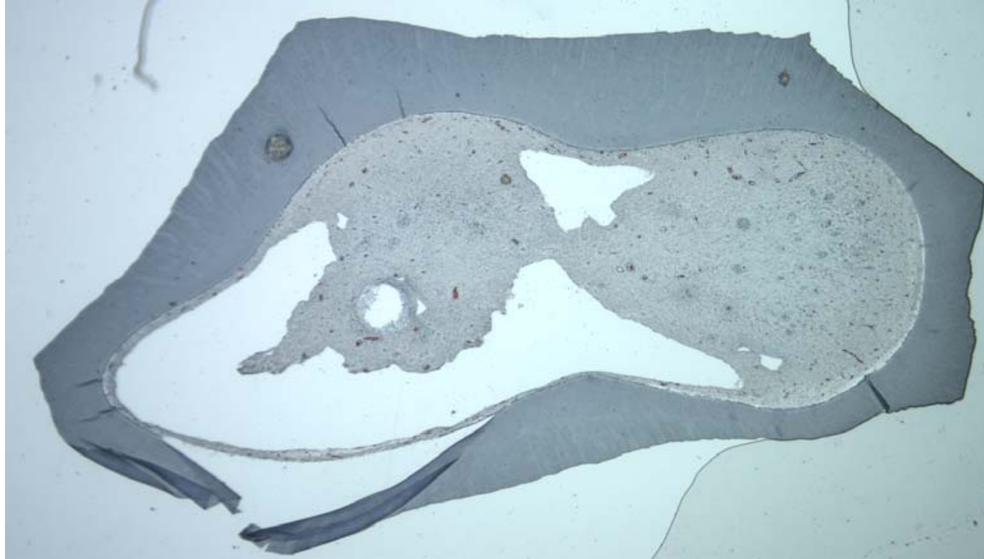

Figure 14. Pulpal voiding. FVIII Immunohistochemical staining of a representative pulp after seven days *in vitro* culture (20x). Pulpal voiding was encountered in the initial tooth slice culturing experiments. It occurred due to pulpal shrinkage upon fixation. Shrinkage created a concavity in the pulp tissue. Histological sectioning through a concave pulp created a void with pulp tissue remaining mostly beneath the pre-dentin layer, with several large voids located centrally.

2. Laboratory Protocols

A. Immunohistochemical staining protocol for Factor VIII with EnVision™ Systems (DakoCytomation, Carpinteria, CA)

1. Bake slides at 37°C for 30 minutes; then bake at 60°C for 30 minutes.
2. Cool slides at RT for 20 minutes.
3. Deparaffinize and rehydration
   a. Xylene 1 3mins
   b. Xylene 2 3mins
   c. Xylene 3 3mins
   d. 100% ETOH 2mins
   e. 100% ETOH 2mins
   f. 95% ETOH 2mins
   g. 95% ETOH 2mins
   h. 75% ETOH 5 dips
   i. DDW 1min
4. Antigen Retrieval

   Immerse slides in 1x Target Retrieval Solution (ref. S1699 DakoCytomation, Carpinteria, CA): 9x DDW. Incubate slides at 90°C in a water bath for 30 mins.

   Allow slides to cool for 20 mins.

   Circle tissue on the slides with a PAP Pen.

   Wash slides with 1x wash buffer (ref. S3006 DakoCytomation, Carpinteria, CA).
5. Endogenous peroxidase blocking

   Place Peroxidase Blocking reagent (ref. K4008 DakoCytomation, Carpinteria, CA) on bench to equilibrate at RT.

Cover tissue sections with Peroxidase Blocking reagent and incubate at RT for 5 mins.

Rinse gently with wash buffer.

6. Primary Antibody

   1:250 dilute Factor VIII (Factor VIII related antigen Ab-1 Rabbit PAb (polyclonal rabbit anti-human Factor VIII) ref. RB-281-A Lab Vision Corporation, Neomarkers, Fremont CA; Antibody Diluent (ref. S0809 DakoCytomation, Carpinteria, CA).

   Add primary antibody to slides and incubate at $4^{o}C$ overnight.

   Rinse gently with wash buffer and remove excess buffer.

7. Peroxidase Labeled Polymer (ref. K4008 DakoCytomation, Carpinteria, CA)

   Add HRP labeled Polymer, rabbit (AEC+), to each slide. Incubate for 1 hour.

   Rinse gently with wash buffer and remove the excess buffer.

8. Chromogen development

   Add AEC+ solution (ref. K4008 DakoCytomation, Carpinteria, CA) to each slide.

   Incubate at RT for 3 mins (time dependent on background stain development).

   Stop reaction by placing the slides in DDW for 5 mins.

9. Counter stain with Hematoxylin

   Place slides in Mayers Hematoxylin (Sigma Aldrich, St Louis, Missouri, MO).

   Rinse under running water to remove excess Hematoxylin.

   Transfer into DDW for 5 mins.

10. Mount slides with aqueous mounting media and cover slides.

11. Seal cover slides with Nail Varnish

B. FGF-2 ELISA Protocol For Cell Culture Supernate Samples (R & D Systems Inc, Minneapolis, MN).

1. Aliquot samples as follows:
    a. 1 ml of DMEM
    b. DMEM + 20% FBS
    c. DMEM + 20% FBS + 10 ng/ml FGF-2
    d. DMEM + 20% FBS + 50 ng/ml FGF-2

    Each total volume of 1ml; giving four samples; allowing for triplication of samples
2. Dilute the two FGF-2 groups by a factor of 100x with plain DMEM (no FBS, antibiotics or any growth factor added) to prevent an overflow reading.
3. Next Steps are preformed using the Human FGF-2 Quantikine Kit (ref. DFB50 R&D Systems)
4. FGF-2 Standard Preparation

    Reconstitute the FGF-2 Standard with 2 ml of the Calibrator Diluent RD5-14. This produces a stock solution of 640 pg/ml. Allow the standard to sit for 15 mins with gentle agitation prior to making diluents
5. Standard Dilution Series

    Using eppendorfs pipette 500 µl of Calibrator Diluent RD5-14 into each tube. Use the stock solution to produce a dilution series (Fig. 15).

6. Assay Procedure (Fig. 16)

    a. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal. Make sure to allow for triplication of each sample in the wells.

    b. Add 100 µl of Assay Diluent (RD1-43) to each well.

    c. Add 100 µl of Standard or sample to each well. Cover with the adhesive strip provided and incubate for 2 hours at RT. Place the plate into the foil pouch and seal it. Record the sample triplication on plate layout sheet provided.

    d. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (approx 400 µl per wash), using a Finnpipette (Fisher*brand*). Tap the well plate, gently on the bench to obtain complete removal of wash buffer, after the last wash. Wash buffer remnants will cause dilution of the assay reagents to be used next, interfering with the reaction giving erroneous results.

    e. Add 200 µl of FGF-2 Conjugate to each well. Cover with an adhesive strip. Incubate for 2 hours at RT and place the plate into the foil pouch.

    f. Repeat the washing as in step d, being careful to wash correctly so as to remove the unbound antibody and wash buffer completely.

    g. In a dark room add 200 µl of Substrate Solution to each well. Incubate for approximately 30 mins at RT.

    h. Add 50 µl of Stop Solution to each well, observing a color change in each well. If the color change does not appear uniform gently tap the plate to ensure through mixing.

i. Determine the optical density of each well within 30 mins of step h, using a microplate reader set to 450 nm

7. Re-calculate the concentration of growth factors in the medium by multiplying each sample reading from the microplate reader by a factor of 100, to allow for dilution (step 2). The standard preparation must be in pg/ml not ng/ml to prevent overflow, as pg/ml concentration is the standard which the microplate reader will use to compare to the triplicated samples.
8. Plot a graph using the data.

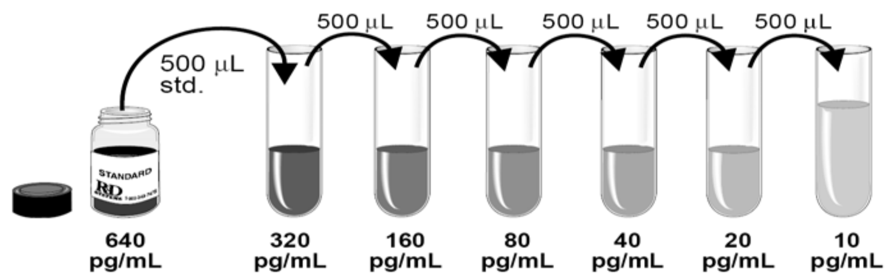

Figure 15. FGF-2 Standard preparation using eppendorfs (R & D Systems).

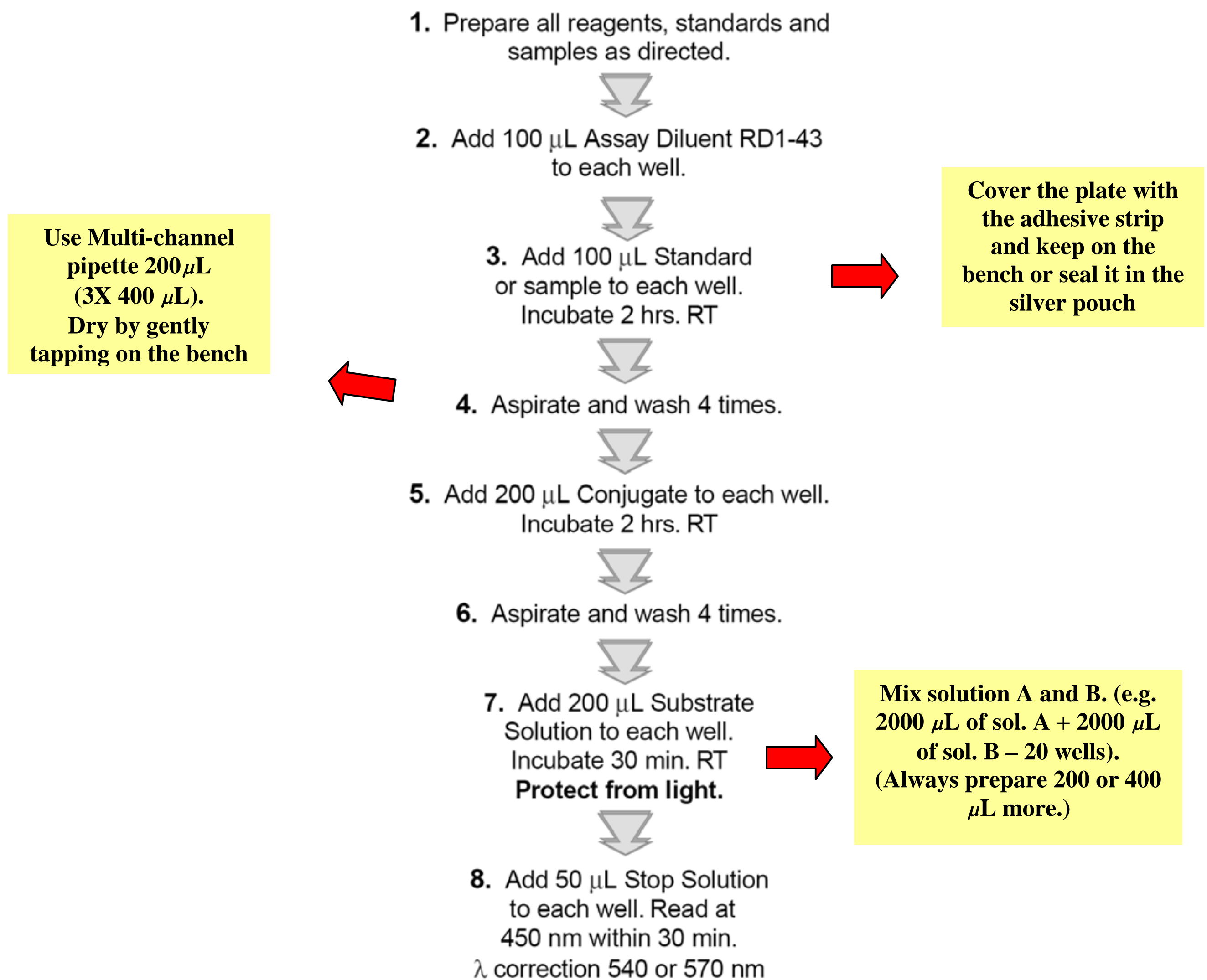

Figure 16. FGF-2 Elisa Assay Summary (R & D Systems).

C. VEGF ELISA Protocol for Cell Culture Supernate Samples (R & D Systems Inc, Minneapolis, MN)

1. Aliquot samples as follows:
    a. 1 ml of DMEM
    b. DMEM + 20% FBS
    c. DMEM + 20% FBS + 10 ng/ml VEGF
    d. DMEM + 20% FBS + 50 ng/ml VEGF

    Each total volume of 1 ml; giving four samples; allowing for triplication of samples
2. Dilute the two VEGF groups by a factor of 100x with plain DMEM (no FBS, antibiotics or any growth factor added) to prevent an overflow reading.
3. Next Steps are preformed using the Human VEGF Quantikine ELISA Kit (ref. DVE00, R&D Systems, Minneapolis, MN)
4. VEGF Standard Preparation

   Reconstitute the VEGF Standard with 1ml of the Calibrator Diluent RD5K. This produces a stock solution of 2000 pg/ml. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions
5. Standard Dilution Series

   Using eppendorfs pipette 500 µl of Calibrator Diluent RD5K into each tube. Use the stock solution to produce a dilution series (Fig. 17). Mix each tube thoroughly before next transfer. Calibrator Diluent RD5K serves as the zero standard (0 pg/mL).
6. Assay Procedure (Fig. 18)

e. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal. Make sure to allow for triplication of each sample in the wells.
f. Add 50 µl of Assay Diluent (RD1W) to each well
g. Add 200 µl of Standard or sample to each well. Cover with the adhesive strip provided and incubate for 2 hours at RT. Place the plate into the foil pouch and seal it. Record the sample triplication on plate layout sheet provided.
h. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (approx 400 µl per wash), using a Finnpipette (Fisher*brand*). Tap the well plate, gently on the bench to obtain complete removal of wash buffer, after the last wash. Wash buffer remnants will cause dilution of the assay reagents to be used next, interfering with the reaction giving erroneous results.
i. Add 200 µl of VEGF Conjugate to each well. Cover with an adhesive strip. Incubate for 2 hours at RT and place the plate into the foil pouch.
j. Repeat the washing as in step d, being careful to wash correctly so as to remove the unbound antibody and wash buffer completely.
k. In a dark room add 200 µl of Substrate Solution to each well. Incubate for approximately 20 mins at RT.
l. Add 50 µl of Stop Solution to each well, observing a color change in each well. If the color change does not appear uniform gently tap the plate to ensure thorough mixing.

m. Determine the optical density of each well within 30 mins of step h, using a microplate reader set to 450 nm

7. Re-calculate the concentration of growth factors in the medium by multiplying each sample reading from the microplate reader by a factor of 100, to allow for dilution (step 2). The standard preparation must be in pg/ml not ng/ml to prevent overflow, as pg/ml concentration is the standard which the microplate reader will use to compare to the triplicated samples.
8. Plot a graph using the data from the microplate reader.

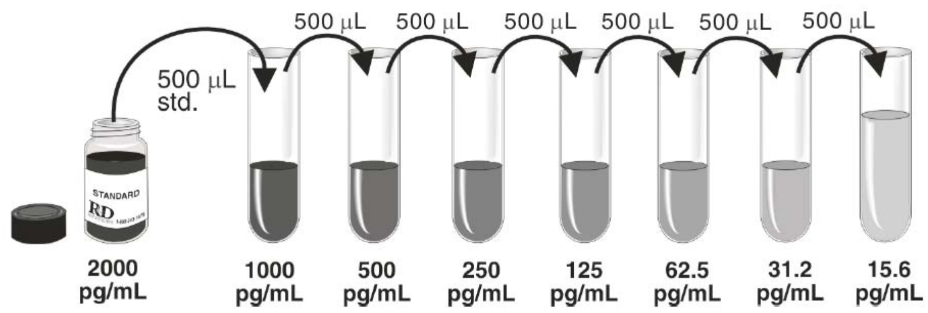

Figure 17. VEGF Standard preparation using eppendorfs (R& D Systems).

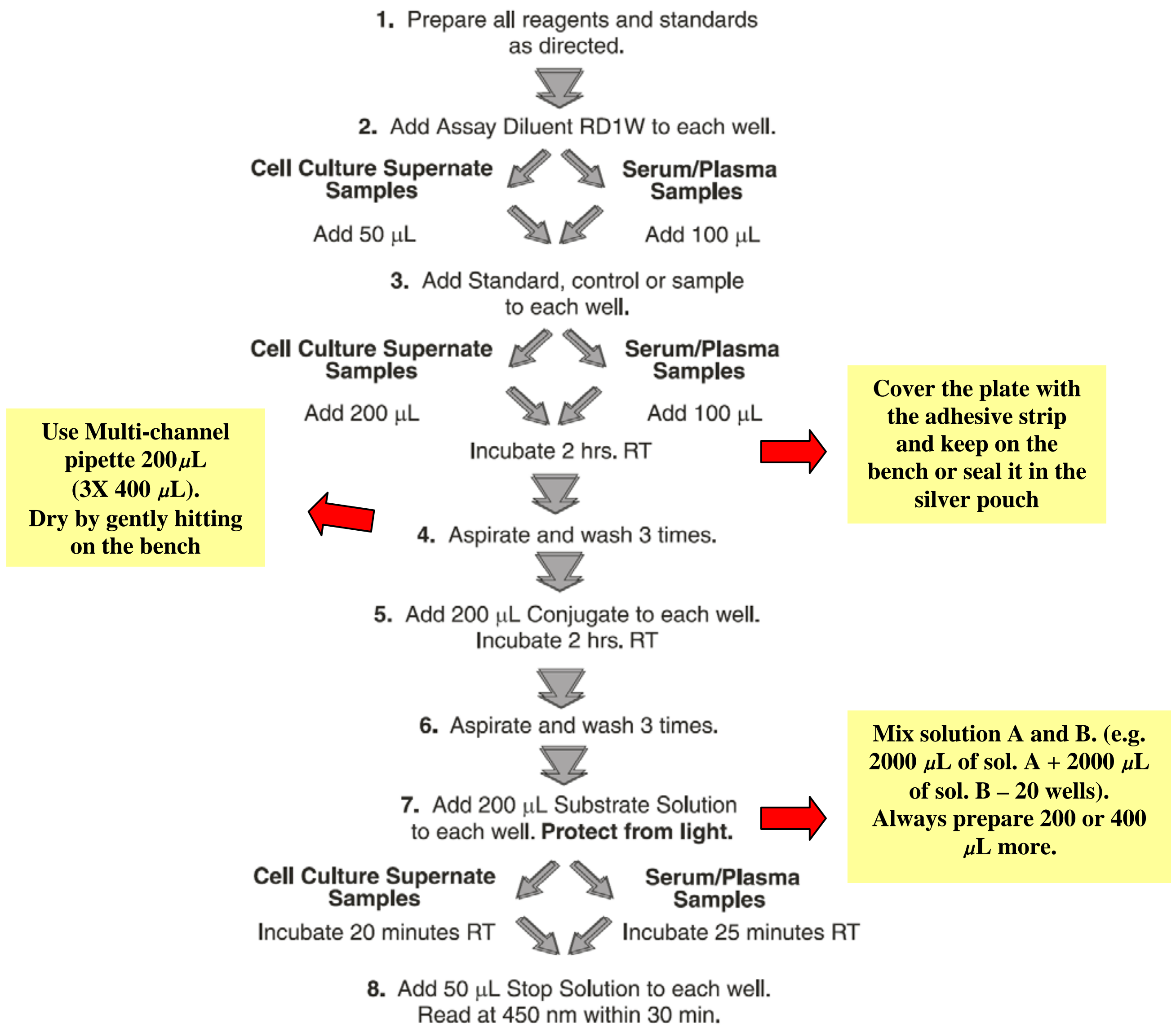

Figure 18. VEGF Elisa Assay Summary (R & D Systems).

D. Demineralization Protocol

1. Following Fixation of the severed human dental pulps in 10% Neutral Buffered formalin (ref. SF100-4, Fisher Scientific) wash the tooth slices three times with PBS 1x (ref. 10010 Gibco).

2. Place four tooth slices (maximum) in a 50 ml BD Falcon™ Tube (ref. 3521956 BD, Biosciences, Franklin Lakes, NJ.).

3. Fill the tube with 40 ml of Decalcifier II (ref 00460 EDTA/HCl; Surgipath). The decalcifier is highly corrosive and is a strong acid. Take precautions to protect against splashes and spillages. Filling the tube with 40 ml leaves space for an air bubble which will assist with fluid flow over the tooth slices, during rotation.

4. Seal the Falcon Tube/s and place them on a rotator (Reciprocating Shaker Model Number: 30153. Barnstead International, Dubuque, Iowa, IA) at RT to create fluid circulation over the tooth slices.

5. 1 mm thick tooth slices should be exposed to the Decalcifier II for no more than 22-24 hours. After this time damage to the pulp tissue may occur, this may impair immunohistochemcial staining.

6. Remove the tooth slices from the Falcon Tube/s and wash with PBS 1x.

7. Trim the demineralized dentin from the periphery of the tooth slices with a razor blade (ref. 94-0451 Safety Razor Blade, Verona, VA), leaving approximately 1mm adjacent to the fixed pulp tissue. During trimming the level of demineralization can be assessed. In general 24 hours of demineralization is sufficient.

8. Place the trimmed tooth slices in a Falcon tube containing 15-30 ml of 70% ETOH.

9. The tooth slices are now ready for histological processing.

E. Acquiring TIFF images, Grid Application and Microvessel Quantification of Severed Human Pulps using Image Pro Plus

Part I: Acquiring TIFF Images

1. Place a single histology slide in the slide mount of the microscope (Eclipse E800, Nikon, Melville, NY).

2. Open Image Pro Plus (version 5.1.2.59 for Windows XP. Media Cybernetics Inc, Silver Spring, MD). Click on Acquire in the drop down menu and scroll to Scan.

3. This will open the Camera (Spot RT, Diagnostic Instruments Inc, Sterling Heights, MI) window. Click on the Live button (top left of the open window). This displays a live image of the histology slide at whatever power the objective lense of the microscope is set to. Images should be acquired on the Brightfield-Transmitted light setting, as seen in the Camera window (bottom).

4. On Live image open window, click on the controls icon (bottom of the Live image window). Click on the drop down menu for Filter Color and scroll to the Clear setting from the current RGB setting. This changes the color setting of the live image, to clear. It is best to use this mode when selecting the correct fields after grid application. This prevents image blurring and allows Image Pro Plus to process the image in a faster fashion. The full chip selection box on the open window must be

selected. Before you acquire an image set the Filter Color to RGB.

5. Click Snap in the open window. This acquires the image and it appears in another window. All pictures taken must be saved in TIFF and not as a JPEG file. TIFF offer better resolution, which is important when counting microvessels from images, obtained using the 200x objective. TIFFs should be converted to JPEG if they will be used in a manuscript or a publication.

Part II: Grid Application

1. Obtain an image as described above but with the 20x objective and save it as a TIFF. A Grid is then applied to the 20x TIFF image (of for example, the complete tooth slice).

2. From the drop down menu, click Process and scroll to Create Mask. On the open window, verify that the grid spacing parameters are set to 160.4 and 100.4. Select Lines in the Objects column and Orthogonal in the Layout column. These specific settings create lined grid boxes, which are the same size as the image obtained with the 200x microscope objective. Specific fields are then selected after the grid is superimposed on the TIFF image.

3. Click Apply, followed by Create Mask and then click OK. From the drop down

menu, click Process and scroll to Image Overlay. Select the image, which the grid will be superimposed on from the Transparency drop down menu. Click Overlay in the open window.

4. Move the grid which was over laid, on the source image. Once it is in the appropriate position click Merge. This merges the grid with the 20x TIFF image in the form of a grayscale picture. Save this image. This can be printed and fields are chosen at random using this printed image. 200x TIFFs are then obtained of these chosen fields, by moving the microscope stage and thus the histological slide to the correct grid location. Use the printed image as a guide (Fig. 7).

Part III: Microvessel Counting using Image Pros Plus Manual Tag

1. Open a saved 200x TIFF that was obtained from a specific grid reference.

2. From the drop down menu, click Measure and scroll to Manual Tag. Manual tagging allows individual microvessel counting and in doing so, a number is applied to each microvessel (red stained by Factor VIII Immunohistochemistry; APPENDIX). This prevents double counting of microvessels as could occur by manual counting.

3. Click on Tag points in the open window. The Add New Points window opens. Click on a microvessel in the 200x TIFF image. This places a number next to the specific

microvessel and now a Total Count is recorded.

4. Total counts from the 200x fields/tooth slice are input into an excel spreadsheet. Data is then input into SigmaStat for statistical analyses (MATERIALS and METHODS).

F. Preparation of Tooth Slices with No Severed Human Dental Pulp Tissue for Implantation into Severely Combined Immunodeficient Mice

Vital, non-carious and non-restored human third molars from patients not older than 30 years of age were collected from the Oral Surgery Department of the University of Michigan School of Dentistry, Ann Arbor, Michigan. After extraction the teeth were immediately placed into sterile Transport Medium (High glucose DMEM (Sigma Chemical Co.) supplemented with 5 ml L-glutamine (Gibco) 200 mM, 5 ml of 10,000 units of penicillin and 10 mg/ml streptomycin (Gibco) and 1 ml Amphotericin B (Sigma) 1.25 mg/l).

The teeth were surface disinfected using a sterile gauze swab soaked in 70% ethanol solution. Excess soft tissue covering the root surface was removed by trimming with a sterile curette.

Preparation of Specimens

Teeth were fixed to a wooden block (3 cm x 3 cm x 1.5 cm) by self curing acrylic (Coldpac, tooth acrylic, Moltoid, Chicago, IL ref 44100) and these blocks were attached to an Isomet Low Speed (Model 650, South Bay Technology, Inc.) saw mount. The teeth were cut into sections of 1 mm thickness with a lapidary blade 303 Series (MK-303 Professional – Mk Diamond Products Inc.) and cooled with sterile PBS-1x-phosphate buffered saline (ref. 10010 Gibco). The sterility of the saw was maintained by washing with 70% ethanol and sterile PBS.

The pulp tissue was removed from each tooth slice with a sterile curette. Once the pulp was removed the pulpal-dentin junction was scrapped with the curette (kindly donated by the

Department of Periodontics and Oral Surgery, University of Michigan School of dentistry, Ann Arbor) so as to completely remove any remaining pulp tissue.

The above procedure was repeated until 12 tooth slices (six tooth slices with no pulp tissue per experiment) with no pulp tissue were deemed suitable for SCID mouse implantation.

Storage of Tooth Slices Prior to Implantation

The 12 slices were then placed into a 50 ml BD Falcon™ Tube (ref 3521956 BD, Biosciences, Franklin Lakes, NJ.) containing 15ml of 70% ETOH. The ETOH storage disinfects the tooth slices prior to implantation.

The day before implantation six tooth slices were removed from the 70% ETOH, in the laboratory fume hood and are washed three times in sterile PBS 1x (Gibco). Each slice is placed into a well of a 6 well plate, containing 5ml of PBS 1x. The well plate is sealed with parafilm (ref. 52858-000 Parafilm M™.) and placed in a refrigerator.

The day of the implantation surgery the tooth slices are removed from the refrigerator and removed from the PBS 1x and are implanted into the dorsal subcutaneous tissues of the SCID mice.

G. IRB and UCCA Protocols

THE UNIVERSITY OF MICHIGAN
OFFICE OF THE VICE PRESIDENT FOR RESEARCH
BEHAVORIAL SCIENCES INSTITUTIONAL REVIEW BOARD
HEALTH SCIENCES INSTITUTIONAL REVIEW BOARD
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Dr. Jaques Nor
Cariology,Restor Sci & Endo
School of Dentistry
1011 N University
1078

Dear Dr. Nor:

The Health Sciences Institutional Review Board (IRB) has reviewed and approved your research proposal involving human subjects. The IRB determined that the research and its procedures are compliant with appropriate guidelines, state and federal regulations, and the University of Michigan's Federal Wide Assurance (FWA00004969 Expiration 6/12/06) on file with the Department of Health and Human Services (HHS).

Please remember that approval must be obtained for changes in procedures or consent document(s) related to your research proposal. If changes are contemplated, they must be approved prior to initiation of the modified procedures.

The approval period for this project is for a period of one year from the approval date listed below, or a shorter period, if specified. *Please note your expiration date.* Approximately three months prior to the expiration date, you will be notified so that your renewal application can be prepared, submitted, and reviewed in a timely manner without interruption in the approval status of this project. You must allow up to six weeks for the review process. **If you allow your approval to lapse, no work may be conducted on this project until appropriate approval has been obtained.**

You are also required to inform the IRB of all unanticipated or adverse events (i.e., physical, social, or emotional injury) as soon as possible after the event. The forms necessary for modifications and adverse event reporting can be obtained on the IRB website at http://www.irb.research.umich.edu.

Sincerely,

Charles J. Kowalski

Chuck Kowalski, Ph.D.
Co-chair, Health Sciences Institutional Review Board

cc: DRDA

PROJECT TITLE: Maintaining slices of human dental pulp in vivo implanted subcutaneously in immunodeficient mice
PROJECT APPROVAL DATE: 12/2/2003 TO EXPIRATION DATE: 12/1/2004
SOURCE OF FUNDS: School of Dentistry, U.S. HHS National Institutes of Health,
IRB FILE NUMBER: H03-00001606-I

To obtain related documents: http://www.irb.research.umich.edu

THE UNIVERSITY OF MICHIGAN

UNIVERSITY COMMITTEE ON USE
AND CARE OF ANIMALS (UCUCA)

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November 6, 2006

Jacques Nor
Cariology, Restorative Sciences, and Endodontics
2309 Dental 1078

Dear Dr. Nor:

The University of Michigan Committee on Use and Care of Animals (UCUCA) has reviewed your Application to Use Vertebrate Animals (Form 8225) referenced below. This project has been approved. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals."

When communicating with the UCUCA Office please refer to the Approval Number referenced below. The appropriate Approval Number must accompany all requisitions for animals and pharmaceuticals.

The Approval Period for your Approval Number is also indicated below. However, the United States Department of Agriculture (USDA) requires an annual review of applications to use animals. Therefore, each year of this application prior to the anniversary of its approval date, you will receive a short Annual Review Form. Your continued animal use approval is contingent upon the completion and return of this form. You will also be notified prior to the expiration of the Approval Period so that your renewal application can be prepared, submitted and reviewed in a timely manner and an interruption in the approval status of this project avoided.

Committee approval must be obtained prior to changes from what is originally stated in the protocol. An amendment must be approved prior to the implementation of the change. Contact the UCUCA Office for further information.

The University's Animal Welfare Assurance Number on file with the NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) is November 14, 2005.

If you receive news media inquiries concerning any aspect of animal use or care in this project, please contact Karl Bates, News and Information Services, 764-7260. If you have security concerns regarding the animals or animal facilities, contact Bill Bess, Director of Public Safety, 763-3434.

Sincerely yours,

Susan Stern, MD
Associate Professor and Chairperson,
University Committee on Use and Care of Animals

cc: DRDA

TITLE: **GENE THERAPY FOR REVASCULARIZATION OF DENTAL PULPS**
APPROVAL PERIOD: **11/2/06 – 11/2/09**
FUNDING AGENCY: **DEPARTMENT**
UCUCA APPROVAL NUMBER: **08754**